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**Title: Gram-negative trimeric porins have specific LPS binding sites that are essential for porin biogenesis.**

**Short title : LPS-Porin Structure**

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## **Abstract**

The outer membrane (OM) of Gram-negative bacteria is an unusual asymmetric bilayer with an external monolayer of lipopolysaccharide (LPS) and inner layer of phospholipids. The LPS layer is rigid and stabilized by divalent cation crosslinks between phosphate groups on the core oligosaccharide regions. This means that the OM is robust and highly impermeable to toxins and antibiotics. During their biogenesis, OM proteins (OMPs) which function as transporters and receptors must integrate into this ordered monolayer while preserving its impermeability. Here we reveal the specific interactions between the trimeric porins of Enterobacteriaceae and LPS. Isolated porins form complexes with variable numbers of LPS molecules which are stabilized by calcium ions. In earlier studies, two high-affinity sites were predicted to contain groups of positively charged side chains. Mutation of these residues led to the loss of LPS binding and, in one site, also prevented trimerization of the porin, explaining the previously observed effect of LPS mutants on porin folding. The first high-resolution X-ray crystal structure of a trimeric porin – LPS complex not only helps to explain the mutagenesis results but also reveals more complex, subtle porin-LPS interactions and a bridging calcium ion.

## **Significance**

Specific and functional interactions between membrane lipids and proteins are increasingly evident across biology. The outer membranes (OM) of Gram-negative bacteria such as *Escherichia coli* are selective barriers formed by complex lipids (lipopolysaccharides; LPS) and outer membrane proteins. The high stability and low permeability of the OM are critical to bacterial growth and pathogenesis. Here, using biochemical and structural techniques, we reveal specific LPS binding sites on OM porin proteins which allow them to stabilize, rather than disrupt, the ordered network of LPS molecules. Furthermore, we demonstrate that one such site is essential for porin assembly in the OM.

## **\body**

### **Introduction**

Specific membrane lipid–protein interactions are increasingly significant in cell biology but, due to their weak or transient nature, often difficult to define (1). The Gram-negative outer membrane (OM) is a highly asymmetric lipid bilayer with lipopolysaccharide (LPS) on the outer leaflet and phospholipids, mainly phosphatidylethanolamine, on the inner leaflet (2). Both the structure of LPS and the bilayer's extreme asymmetry are unusual for biological membranes and their combined effect upon the integral membrane proteins embedded in the OM have not been clearly investigated. The role of the OM is to create a robust and tight barrier towards the external environment such that transport across it is highly regulated by the outer membrane proteins (OMPs) it contains. Uniquely this also applies to hydrophobic molecules, for which the polar part of the LPS poses a significant permeation barrier. Thus a tight interaction between the LPS barrier and the inserted OMPs is likely to be essential to maintain OM impermeability. With the rise of antimicrobial resistance it has become increasingly clear that the OM affords Gram-negative bacteria an additional and effective means to withstand antibiotic therapy (3). Several lines of evidence have indicated that there is a close and specific interaction between OMPs and LPS. In early studies of matrix porin (OmpF), LPS co-purified with the protein and a ratio of 3:1 LPS:OmpF was determined (4, 5). It was also proposed that LPS was required for full ion channel activity (6, 7). LPS binding to OmpF was later analyzed by free-flow electrophoresis (FFE) which separated four isoforms clearly visible as ladders on SDS-PAGE (8). All forms contained approximately 1 mole of tightly bound LPS per trimer with three additional isoforms containing 1, 2 and 8 mol per trimer of loosely bound LPS respectively so that the highest stoichiometry observed was nine per trimer or a 3:1 LPS:OmpF molar ratio. Interestingly, analytical centrifugation of samples which produced ladders on SDS-PAGE showed a single species in solution and addition of 3 mM EDTA partially removed the loosely bound LPS (8).

The purified isoforms were used to form OmpF 2D crystals observed by negative stain electron microscopy. A protrusion on the central three-fold axis of the trimer was linked to the single, tightly bound, molecule of LPS per trimer. Less distinct protrusions at the

perimeter of each trimer were observed in samples with loosely bound LPS and thus ascribed to sites of partial LPS occupation (9). A similar central location of a single LPS molecule was also proposed for the structure of PhoE porin derived from cryoelectron microscopy (10). The samples with 1 mol of tightly bound LPS formed 3D crystals suitable for X-ray crystallography but the structure has not been reported (8). Porin from *Yersinia pseudotuberculosis* was found to show two or three different binding sites depending upon the form of LPS acylation (11). The first trimeric porin structure solved to high resolution was from *Rhodobacter capsulatus* (12) and neither this, nor the later *E. coli* OmpF and PhoE structures (13), showed any evidence of bound LPS. Furthermore none of the structures of trimeric porins published since show identifiable LPS on the three-fold axis or on the external surface of the barrel (14) and this is probably due to the stringent removal of LPS in many purification procedures (15). By *in vitro* folding in the absence of LPS we were able to confirm earlier data (16) that LPS-free OmpF trimers show identical structural and electrophysiological properties to those which folded *in vivo* in the presence of LPS in the OM (17, 18).

The first high-resolution structure of an LPS-OMP complex was that of the *E. coli* siderophore transporter FhuA (19). It revealed for the first time the non-covalent interactions that could stabilize a tight association of LPS with protein. The lipid A acyl chains form van der Waals interactions with the membrane-exposed hydrophobic surface of the protein, whilst basic amino acids form salt bridges with the phosphate groups on LPS. The LPS-OMP structure enabled a bioinformatic search of the structural database which led to the definition of a likely LPS binding site, based upon a four-residue motif, in a variety of OM and innate immunity proteins (20). An obvious limitation of the bioinformatic approach is that the LPS binding site definition was based on the structure of a single LPS-OMP complex, since FhuA is the only OMP for which structures with bound LPS have been reported. A crystal structure of the omptin protease Pla from *Yersinia pestis* showed density consistent with LPS acyl chains, but no density for the polar part of the glycolipid was observed (21, 22). Using the predictions from FhuA we predicted likely LPS binding sites on the OmpF trimer and showed that these corresponded to changes in 2D crystal structure when the antibacterial toxin colicin N displaced tightly bound LPS from the OmpF trimer (18).

In addition to its role in creating the OM barrier, LPS is also the environment into which trimeric porins are inserted and folded. It is well established that reduction in the amount or size of LPS molecules severely inhibits trimer formation (7, 23-25) whilst LPS and divalent cations are able to induce folding of porins *in vitro* (26). In recent years the discovery and characterization of the relevant periplasmic chaperones and  $\beta$ -barrel assembly machinery (BAM) complex have revealed in significant detail the protein components of the OMP biogenesis process (27). By contrast, how the LPS guides the folding and trimerisation of porins is less clear.

Here we show by mutagenesis, small angle X-ray and neutron scattering and X-ray crystallography that there are at least two specific binding sites for LPS per porin monomer and that one of these is both calcium dependent and essential for the assembly of trimers in the OM. The results reveal that trimeric OMPs form a specific, tight complex with LPS that maintains the permeability barrier of the OM.

## Results

**SDS-PAGE can resolve OmpF-LPS Complexes.** The addition of LPS (Figure 1A) to LPS-free *in vitro* folded OmpF trimers (18) creates, on SDS-PAGE, a characteristic ladder of increasing molecular mass due to the slower mobility of LPS-bound OmpF (Figure 1B) (8). Moreover, the results confirm that LPS-OmpF complexes form with a range of stoichiometries within each sample and are stable under SDS-PAGE conditions without boiling (18). Moreover these ladders resemble those observed in preparations of native OmpF from *E. coli* OM fractions if LPS is not intentionally removed (18).

**Divalent cations stabilize OmpF-LPS complexes.** Since divalent cations are known, via interactions with phosphate groups, to stabilize LPS in the OM (28) and in protein complexes (8), we tested whether the complexes here were similarly stabilized. The addition of 5 mM  $\text{CaCl}_2$  or  $\text{MgCl}_2$  increased the resolution of bands displayed on SDS-PAGE by the complexes formed with a 5:1 ratio of Ra-LPS to OmpF. Addition of 5 mM EDTA reduced but did not abolish the ladder (Figure 1C), implying the presence of some non-calcium bridged LPS-OMP complexes.

**OmpF binds to all LPS types tested including minimal Lipid A.** *In vitro* folded LPS-free OmpF was mixed with LPS molecules of increasing length, starting with lipid A (Figure 1D), at a molar ratio of five LPS to one OmpF monomer (15 LPS per trimer). The shift in mobility of the LPS bound forms is proportional to the size of the LPS molecule added (29). Even though with Lipid A a ladder cannot be resolved, a clear retardation indicates that even this minimal LPS molecule forms stable complexes with OmpF. Thus the multiple bands which form the long LPS-induced ladder on SDS-PAGE are probably complexes containing different ratios of LPS:OmpF, stabilized by low ambient levels of divalent cations recruited from the LPS and buffer solutions. To further understand the ladder of complexes observed in SDS-PAGE we attempted to saturate the LPS binding sites by adding higher molar ratios of LPS. We used the largest rough LPS molecule, Ra-LPS, to provide the clearest shift and added up to a tenfold molar ratio (30 LPS per trimer). Increasing ratios of LPS led to increasingly higher ladders with no saturation nor clear population of a preferred complex (Figure 1E).

**Mutagenesis indicates possible LPS binding sites of OmpF.** From the structure of FhuA (19) and subsequent bioinformatic analysis of the structural database (20) it was predicted that the LPS binding site(s) on OmpF should consist of about four basic amino acid residues facing the membrane near the OM extracellular surface where they can interact with the phosphate groups on the lipid A core (18). Ten arginine and lysine side chains form a belt at the correct height on the OmpF surface for LPS binding and these can be divided into two distinct groups which we termed sites A and B (Figure 2A). A series of site A mutations were designed, purified from the OM and tested on SDS-PAGE for their ability to purify as a complex with the natural LPS from their host *E. coli*. We initially chose glutamine to replace the lysine and arginine chains in order to minimize the changes to the local structure. Single mutants showed marginally decreased ladders but significant effects were only seen with double and triple mutations (Figure 2B).

**LPS binding at Site B is essential for stable trimer formation *in vivo*.** To comprehensively study the roles of the two sites we performed complete mutagenesis of all 4 basic residues in the A site and all 6 in the B site (for details of mutated residues see Figure 2A) to polar uncharged glutamine (A-Gln and B-Gln), negatively charged

glutamate (A-Glu, B-Glu and both sites AB-Glu) and non-polar alanine (A-Ala, B-Ala, AB-Ala). Each mutant protein was purified from the *E. coli* OM and trimer formation assessed by SDS-PAGE. Only the site A mutants (site B intact) were able to mature and form trimers in the OM. In all the site B mutants the expressed OmpF remained as monomers (Figure 3 A-C). To test whether site B mutants could still form trimers *in vitro*, we purified the most radical mutants (lysine/arginine to glutamate substitutions which reverse the charge of the sites) as inclusion bodies by expressing them without signal sequences (17). The inclusion bodies were solubilized in urea and ran on SDS-PAGE as monomers with slightly altered mobilities (Figure 3D). Following *in vitro* folding both the individual A-Glu and B-Glu site mutants formed trimers but the double site mutant AB-Glu did not fold properly (Figure 3E). This showed that OmpF lacking site B can still fold to form SDS-stable trimers *in vitro*. The *in vitro* folded mutant proteins A-Glu and B-Glu were then mixed with a 5:1 ratio of Ra-LPS:OmpF and analysed on SDS-PAGE. Each mutant protein showed simpler ladders than WT and bound Ra-LPS in a calcium-dependent manner. The effect of EDTA on the OmpF-LPS ladders was more pronounced in the A-Glu compared to the B-Glu mutant (Figure 3F).

**Structure of OmpF-LPS complexes in solution.** We performed dynamic light scattering (DLS) measurements on OmpF-LPS complexes in 1% SDS. This revealed a narrow distribution of radii slightly larger than OmpF alone (Figure S1) and is consistent with a population of isolated trimers. This pattern only changed to large aggregates when 10 or more molecules of LPS were added per trimer in calcium-containing solutions. Small Angle X-ray scattering (SAXS) was then employed to more accurately measure the overall dimensions of OmpF-LPS-dodecylmaltoside (DDM) micelles. Recently, it was shown that estimates of membrane protein structure can be derived from SAXS even when there is extensive scattering from detergent micelles (30). This enabled us to calculate a maximum size ( $D_{\max}$ ) of about 130 Å for the OmpF-LPS complex. (Figure S2). We then used neutron scattering which can resolve different components of biological complexes (31). Neutron scattering is especially sensitive to the different neutron scattering length densities (nSLDs, analogous to a neutron refractive index) of hydrogen and its isotope deuterium. Thus we prepared deuterated OmpF (d-OmpF) with an nSLD equal to that of 81% v/v D<sub>2</sub>O (the “match point”) (Figure S3A). We then formed complexes of d-OmpF and normal hydrogenous



Ra-LPS (h-Ra-LPS) which has a match point of 27% v/v D<sub>2</sub>O (32). We then collected small angle neutron scattering (SANS) data from the d-OmpF/h-Ra-LPS complexes, in a range of D<sub>2</sub>O concentrations to highlight the separate contributions of protein and LPS to the complex (Figure 4A). The measured nSLD of the complexes has a match point of 70% v/v D<sub>2</sub>O which most closely matches that expected for a complex with 2 LPS per trimer (Figure S3 A,B). The scattering curves were used to calculate pairwise distance distributions (P(r) plots) (Figure 4B). Small angle scattering data collected from OmpF-LPS complexes in 13%, 27% (where LPS is completely invisible to neutrons) and 41% D<sub>2</sub>O were dominated by d-OmpF scattering and resembled previous d-OmpF data (32). By measuring SANS in 77% D<sub>2</sub>O, close to the contrast match point for d-OmpF, we obtained data dominated by LPS scattering. However, since each trimer may only contain 6 Ra-LPS molecules which have a total mass of 27 kDa compared to the 120 kDa OmpF trimer, the scattering under these conditions is much weaker than when the protein dominates the signal (Figure 4A, Table 1). Nevertheless, in this case the LPS scattering profile was completely different and resulted in a P(r) plot with two peaks. This profile can be explained by scattering centers (LPS molecules) on the outside of the complex, separated by the diameter of the trimer, as seen previously with the complex of colicin N with OmpF (32). The first peak around 20 Å corresponds to distances within each group of bound LPS molecules at sites A and B. The second peak, centered at 90 Å, corresponds to the set of LPS-LPS distances across the trimer. We further analyzed the distribution of LPS using the Stuhrmann plot (33), the square of the radius of gyration ( $R_g^2$ ) versus the inverse of the contrast between the scattering object and the solvent at different D<sub>2</sub>O concentrations ( $1/\Delta\rho$ ) (33) (Figure 4C). Using the program MULCh, we fitted the data to the equation

$$R_g^2 = R_m^2 + \frac{\alpha}{\Delta\rho} - \frac{\beta}{\Delta\rho^2}$$

where  $R_m$  is the  $R_g$  of an equivalent complex with a homogenous scattering density,  $\alpha$  relates to the difference in distribution of the scattering densities and  $\beta$  to the difference in center of mass of the scattering densities (33). The result was the line in Figure 4C with the following parameter values  $R_m^2 = 1534 \pm 37 \text{ Å}^2$ ,  $\alpha = -863 \pm 83$ ,  $\beta = 324 \pm 51$ . The negative  $\alpha$  value indicates that the lower nSLD component (LPS) has a larger radius of gyration and is thus at the periphery of the complex. Finally, we computed

theoretical scattering curves for a number of OmpF-LPS complexes using the program CRYSON (34) and compared them with our experimental data (Figure S3C). The data fitted OmpF models with LPS occupying either two (using the positions suggested by the OmpE36-LPS structure) or three (with an additional LPS next to site B) Ra-LPS per trimer with similar statistics. The fits were improved when the sugar head groups were modelled as bent away from the trimer (Figure S3C).

**Structure determination of the OmpE36-LPS complex.** To provide support for the biochemical data we overexpressed and purified several Enterobacterial porins from the OM of *E. coli* with the goal of obtaining a structure with bound LPS. We initially focused our efforts on *E. coli* OmpF, but did not succeed in obtaining well-diffracting crystals. We then extended our focus to the OmpF and OmpC orthologs from other, closely related enteric bacteria, including those from *Klebsiella pneumoniae* and *Enterobacter cloacae*. The OmpC ortholog from *E. cloacae* (OmpE36) gave well-diffracting crystals. The structure was solved by molecular replacement (MR) with *E. coli* OmpC as a search model (PDB ID 2J1N; 66 % sequence identity), using data to 1.45 Å resolution. There are two porin trimers in the asymmetric unit, arranged as a double layer of 2D crystals (Figure S4). Inspection of the initial difference maps showed clear density for large molecules bound to the outside surface of the trimer, which could be unambiguously assigned to LPS (Figure 5 and Figure S4).

A total of four LPS molecules are bound to each OmpE36 trimer (Figure 5A). One LPS molecule (LPS A) is bound in each groove between two monomers, corresponding to site A (Figure 5A). In addition, one OmpE36 monomer per trimer contains an additional LPS molecule at a position corresponding to site B (LPS B; Figure 5A and Figure S4). This particular LPS molecule is sandwiched by a symmetry-related protein molecule (Figure S4), providing a possible explanation for why there is only one OmpE36 monomer with two molecules of bound LPS. In addition to LPS A and B, there is one other LPS molecule within the asymmetric unit. This molecule (denoted LPS C) is relatively poorly ordered and also bridges to a crystallographic symmetry mate (Figure S4). We have previously observed the ability for LPS to promote 2D crystallization of *E. coli* OmpF trimers and the bridging positions of the site B (and possibly site C) LPS molecule helps explain this behavior (35).

For the LPS A and B molecules, complete density is visible for the lipid A moiety as well as for both Kdo (3-deoxy-d-manno-oct-2-ulosonic acid) residues, *i.e.* for Re LPS. Beyond the Kdo<sub>I</sub> moiety, LPS B has clear density for the GMH (L-glycero-D-manno-heptopyranose) and part of the Rc moieties (Figure 1A and Figure S5), but beyond GMH the density is of insufficient quality to allow building of a reliable model. For the GMH itself, density is visible only for the mannopyranose ring. For LPS A, no model can be built beyond the Kdo residues. The likely reason for the relatively poor density is that beyond the Kdo moieties, the LPS sugars bend away from the protein and hence do not form many interactions. This is supported by the improvement of fits of SANS data to model complexes when the Ra-LPS head groups are bent away from the protein in the detergent micelle (Figure S3C). Interestingly, the density in the lipid A region reveals that both LPS A and B are hepta-acylated (Figure S5), which is in contrast to the hexa-acylated LPS that is thought to dominate in *E. coli* and which is also observed in the FhuA structures.

In addition to numerous van der Waals interactions between the lipid A acyl chains and the hydrophobic exterior of the barrel, LPS A and B make a number of polar interactions with residues of OmpE36, including both salt bridges and hydrogen bonds. For LPS A, salt bridges are present between Lys198 and the GlcN<sub>II</sub>-4P of lipid A and the carboxylate of Kdo<sub>I</sub>, as well as between Arg213 (OmpF K210) and GlcN<sub>II</sub>-4P (Figure 6). The configuration of donors and acceptors suggests that these salt bridges are dynamic and not all present all the time. This may explain why, in spite of few clearly resolved salt bridges, the removal of lysine and arginine residues still reduces LPS binding to OmpF. A final, presumably stable salt bridge is present between Lys152 and the carboxylate of Kdo<sub>II</sub>. Hydrogen bonds are present between the Asp174 side chain and GlcN<sub>I</sub> and GlcN<sub>II</sub> and between the peptide backbone of several residues (Glu159, Arg199 and Ser201) and Kdo<sub>II</sub> (Figure 6). For the LPS B molecule, density consistent with a metal molecule is present between the LPS and OmpE36. Analysis with the CheckMyMetal (CMM) server (47) assigns this metal with high confidence as a calcium ion. Since no metals were added during purification and crystallization the calcium must have been co-purified from the *E. coli* OM. The ligands of the calcium are the side chains of Asn239 and Asn250 as well the backbone carbonyl of Asn210. In addition, four functional groups on Kdo<sub>II</sub> provide ligands to the calcium (Figure 5F and Figure 6). The calcium ion most likely stabilizes the LPS-porin interaction

substantially. In addition to the calcium-mediated interactions, there are also hydrogen bonds between the calcium-liganding residues and Kdo<sub>II</sub>, between Tyr238 and GlcN<sub>II</sub>-4P and lastly between Glu215 and GlcN<sub>I</sub> and GlcN<sub>II</sub> (Figure 6). Thus, the three Asn residues have dual roles in that they hydrogen bond to the Kdo<sub>II</sub> directly but also coordinate the calcium ion that interacts with Kdo<sub>II</sub>. Our work therefore reveals for the first time the structural basis of the previously observed calcium-dependent LPS-porin binding.

## Discussion

The high-resolution X-ray crystal structure of the porin OmpE36 from *E. cloacae* defines two binding sites for LPS molecules per porin monomer on the exterior surface of the barrel. The crystal structure confirms that the areas of density previously observed by electron microscopy correspond to LPS (8, 18). Likewise, the neutron contrast matching enabled us to observe solely the LPS scattering in the OmpF-LPS complexes, and the resulting data fit to a peripheral distribution of groups of LPS molecules around the trimer. The fitted distances between the scattering centers fit well to measured distances in the crystal structure between LPS A-B (~20 Å) and LPS A-A (~90 Å). The structure also agrees with our biochemical and biophysical data for *E. coli* OmpF, which is not surprising given the high sequence similarity between both porins (Figure S6 and Figure S7). Taking the sequence and structural similarity argument further, we predict that LPS binding sites are conserved in Gram-negative bacterial porins. Why then has no porin structure with bound LPS been reported previously despite a large body of structural work? One possible reason is that, based on SDS-PAGE and previous Free Flow Electrophoresis analysis (8), preparations of porins are heterogeneous with respect to their bound LPS. This is even the case for LPS added *in vitro* (Figure 1E) and will likely be exacerbated with protein isolated from the OM. Moreover, the extended ladders observed on SDS-PAGE, when using samples equilibrated with large excesses of LPS, suggest that there may be more than two sites per monomer. Another reason likely lies in crystal packing constraints. The LPS-OmpE36 structure reveals that whereas LPS A is present in all three grooves between porin monomers, LPS B is only observed at one of three possible sites. This LPS molecule is sandwiched by a monomer from a crystallographic symmetry-related neighbor, likely stabilizing it within the crystal. As can be appreciated from Figure S4,

the crystal packing is not compatible with LPS being present at the other two B site positions. Thus, the exceptionally large size of the LPS head group creates tight constraints on the possible packing within the crystal, which together with preparation inhomogeneity provides an explanation for why porin-LPS complexes have proved difficult to crystallize previously. Indeed, crystallization of several other porins from *Enterobacter* and *Klebsiella* spp. purified by us following the same procedure has not yet yielded structures with bound LPS.

**Comparison of LPS binding in FhuA and OmpE36/OmpF.** The determination of the OmpE36-LPS structure allows for the first time a comparison with FhuA-LPS, for which 11 coordinate files are present in the PDB that all show one LPS molecule bound to the same site. For FhuA, basic residues dominate the polar interactions with the bound LPS. No fewer than six different lysine and arginine residues interact with the LPS inner core via salt bridges and hydrogen bonds, whereas only a few non-charged residues of FhuA interact with the LPS molecule. The dominance of basic residues in LPS binding formed the basis of the proposed four-residue LPS binding motif (20) that allowed prediction of LPS binding sites on *E. coli* OmpF (18). Despite the successful prediction, however, there are substantial differences in LPS binding between the two proteins. First, the dominance of basic residues in LPS binding is much less pronounced in OmpE36 compared to FhuA. Three basic residues interact with LPS A in OmpE36 (Figure 6), which in *E. coli* OmpF may be reduced to two (Figs. S6, S7). By contrast, only one basic residue (Arg213) interacts with LPS B in OmpE36 (Figure 5). In OmpF, Lys210 is at the same position as Arg213, but since the lysine cannot interact with both LPS A and B at the same time it is not clear how much Lys210 contributes to binding of each of the LPS molecules in OmpF. Molecular dynamics simulations could likely illuminate this issue. Other than the possible involvement of Lys210, only one other basic residue likely interacts with LPS B in OmpF. This is Arg235, which occupies the same position as Tyr238 in OmpE36 and is likely to interact with GlcNII-4P (Figure 6 and Figure S7). The second difference between LPS binding is the absence of calcium-mediated interactions in FhuA-LPS. The original FhuA-LPS complex structure (PDB ID 2FCP; (19)) contained a putative nickel metal ion bound to one of the phosphates of LPS that did not contribute to the binding to FhuA. However, other FhuA-LPS structures do not show any metal-mediated interactions between the protein and LPS, and we can therefore conclude that divalent

metals do not play a role in the FhuA-LPS interaction. The final major difference in LPS binding between the two OMPs lies in the role of the Kdo<sub>II</sub> moieties. In FhuA-LPS, Kdo<sub>II</sub> points away from the protein and contributes just one interaction to the binding to FhuA (a salt bridge between Arg384 and the Kdo<sub>II</sub> carboxylate). In sharp contrast, in both LPS A and B the Kdo<sub>II</sub> forms the interaction hub with OmpE36 (Figure 6). This is especially true for LPS B, for which Kdo<sub>II</sub> mediates a number of metal-mediated and direct interactions with the porin (Figure 6). It is therefore clear that OMPs can interact with LPS in different ways, and an expanded database incorporating the LPS-OmpE36 data should enable a better prediction of LPS binding sites on OMPs.

**Role of metal in LPS binding.** The site B LPS reveals the molecular basis of the calcium-dependent porin-LPS interaction (Figure 1C) (8). The extended ladders of OmpF-LPS observed on SDS-PAGE when using samples equilibrated with large excesses of LPS occur only in the presence of calcium, indicating (i) that there may be additional B-type sites for OmpF-LPS interactions and (ii) that calcium mediates LPS-LPS interactions leading to an additional row of associated lipids surrounding the complex. The reduction of the size of the LPS-OmpF ladder by EDTA treatment is consistent with only the A sites being occupied by LPS in the absence of calcium, as suggested by the metal-independent binding of LPS A from the OmpE36 structure. Magnesium has also been shown to stabilize LPS-LPS interactions and thus divalent cations are critical for both LPS-LPS (28) and LPS-porin interactions, explaining why EDTA can destabilize the OM sufficiently for small proteins such as lysozyme to penetrate into the periplasm. The importance of divalent metal ions for stabilization of the LPS monolayer is a logical consequence of the presence of many negatively charged phosphate groups (28), which without neutralization would be repulsive and render the OM highly unstable.

**The essential role for LPS in OM structure and function.** The intricate interactions between OmpF and LPS revealed here explain how these very different molecules combine to maintain OM integrity. The porin-LPS interface is likely to be rigid and thus regions of high porin density, observed by electron microscopy (37, 38) and AFM (39) are likely to be robust non-fluid structures with the LPS having very limited diffusion. In an AFM study Schabert and Engel (40) showed that the addition of loosely bound LPS changed 2D crystals from rectangular to hexagonal whilst EM (24) and AFM (39)

studies of bacterial outer membranes reveal quasi-crystalline arrangements of porins and LPS *in vivo*. Furthermore, OmpF stabilized by the polymeric detergent Amphipol can be rapidly induced to form dense 2D arrays by the addition of excess LPS (35). These data, combined with the specific interactions revealed here, explain how porins and LPS form tight assemblies on the surfaces of Gram-negative bacteria that preserve the necessary stability and impermeability of the OM. Light microscopy on live cells using fluorescently labelled outer membrane proteins has provided the best insights into the arrangement of stable assemblies within the outer membrane. In one report it was shown that whilst trimeric porins form relatively immobile regions, monomeric proteins such as BtuB show faster diffusion expected of individual proteins (41). More recently it was shown that assemblies of trimeric OMPs containing trapped BAM complexes are created near the cell midline and stay intact over several cell division cycles to finally end up at the cell poles (42). The trimeric porins are likely to form stable ordered protein-LPS complexes at the heart of these long-lived assemblies.

While being a barrier for large and relatively hydrophobic antibiotics such as macrolides, the OM is also a target for antimicrobial molecules such as polymyxin and colicins. Polymyxins are strongly cationic lipopeptides that are likely able to replace the divalent cations bound to LPS, thus gaining access to the core of the membrane. The large antibacterial protein colicin N was recently shown to use the inner core region of LPS as its specific OM receptor and thus must be able to access the regions of the LPS molecule close to the hydrophobic core (43). In fact, colicin N is the only molecule known to displace tightly bound LPS from the outside of the OmpF trimer (18).

Data gathered over many years have indicated an important role for LPS in the *in vivo* maturation of porins (7, 23, 25, 44-46). Here we provide support for these observations by showing that the successful *in vivo* OM biogenesis of OmpF requires the presence of an intact LPS B site at the periphery of the trimer. By contrast, the successful production of functional LPS-free OmpF by folding into detergent micelles suggested that LPS was not an essential component for OmpF *in vitro* (17, 47). OMP biogenesis is mediated by the BAM ( $\beta$ -barrel assembly machine) complex which accepts unfolded proteins from periplasmic chaperones (48) and mediates the formation of  $\beta$ -barrels in

the OM via a mechanism that is not yet well understood (49-51). How LPS binding at site B affects OmpF biogenesis is therefore not clear, but the fact that this site is not required for *in vitro* folding suggests that the specific LPS B-OmpF interaction stabilizes an intermediate during the BAM-mediated biogenesis of porin trimers in the OM. Future work will be required to establish the extent to which LPS specifically interacts with other OMPs and whether such interactions, analogous to OmpF, directly affect OMP biogenesis *in vivo*. Such data will enable us to better understand the stability and dynamics of the OM, in particular its role as a barrier to antibiotics.

## Methods

**OmpF production.** OmpF mutants were expressed from the plasmid pMS119 encoding the desired mutant *ompF* gene and purified from the OM of *E. coli* BZB1107 (*ompF::Tn5*) as described previously (32). Transformed cells were grown at 37°C in LB (Luria-Bertani) medium supplemented with 100 µg/ml ampicillin, 30 µg/ml kanamycin and 0.05% (v/v) glucose in order to inhibit the expression of another OMP, LamB. PhoE porin expression is suppressed by the phosphate present in the medium. The parental strain is naturally devoid of OmpC. When the OD<sub>600</sub> of the cell culture reached 0.6, IPTG (final concentration of 1 mM) was added to induce protein expression and cells were grown for a further 3 h. Cells were harvested by centrifugation at 8,000 g for 10 min at 4°C.

**Production of deuterated OmpF.** The deuterated OmpF was produced from *E. coli* BE3000 (15, 32). Cells were first adapted onto a H<sub>2</sub>O based, solid minimal medium plate followed by growth on a 85% D<sub>2</sub>O minimal medium plate (52). Once colonies grew on the plate (which normally takes a few days due to the slower growth of the cells), selected larger colonies were grown in 50 ml of 85% D<sub>2</sub>O minimal liquid medium. Once growth was established overnight, these cells were inoculated at 1:20 ratio into 2 x 50 ml of fresh 85% D<sub>2</sub>O minimal liquid medium. This step was repeated three times in order to increase the initial growth rate. Cells were harvested by centrifugation at 8,000 g at room temperature and resuspended into 10 ml of fresh 85% D<sub>2</sub>O minimal liquid medium. 10 ml of cell culture was inoculated into 1.5-litre of minimal medium in a 2 liter Minifors fermenter (INFORS) at 37°C using glucose as a carbon source. During cell growth, a pH of 7.3 was maintained by adding 10% (w/v)



NaOH. The measurement of OD<sub>600</sub> was performed to monitor the cell growth. When the OD<sub>600</sub> reached at least 10.0, the cells were harvested by centrifugation at 8,000 g for 10 min at 4°C.

**Purification of OmpF.** Hydrogenous and deuterated forms of OmpF were purified using the same procedures. The cell pellet was resuspended in 20 mM sodium phosphate, pH 6.8 and supplemented with DNase (10 µg/ml) and RNase (10 µg/ml). The cells were lysed by sonication on ice for 15 min. To remove unbroken cells and cell debris, the suspension was centrifuged at 3,000 g for 15 min. After removing the pellet containing unbroken cells and cell debris, the supernatant was centrifuged at 40,000 rpm for 1 h in a 45 Ti rotor on a Beckman L7-80 ultracentrifuge in order to isolate the membrane pellet. The membrane pellet obtained was resuspended in wash buffer (20 mM Tris, 2% (w/v) SDS, pH 7.4) and then homogenized at 55°C for 1 h. The solution was centrifuged at 40,000 rpm for 1 h in a 45 Ti rotor on a Beckman L7-80 ultracentrifuge and the supernatant was discarded. This wash step was repeated twice. The extraction of OmpF from the remaining membrane pellet was carried out by incubating and homogenizing the pellet in wash buffer with additional 500 mM NaCl. After incubation for 1 h at room temperature, the extract was centrifuged at 40,000 rpm for 1 h in a 45 Ti rotor on a Beckman L7-80 ultracentrifuge. The extraction was repeated once to increase the yield of OmpF. The supernatant containing SDS-solubilized OmpF was dialyzed against 5 mM NaHCO<sub>3</sub>, 0.1% (w/v) SDS overnight at 37 °C. Precipitation of OmpF (to concentrate or change detergent) was achieved by mixing cold ethanol, pre-chilled at -80 °C, with OmpF samples to give a final ethanol concentration of 90% (v/v). This solution was then incubated at -20 °C overnight. To isolate the precipitated OmpF pellet, the solution was centrifuged at 17,000 g for 1 h. The OmpF pellet was dried under a stream of air. OmpF was recovered by resuspension in 20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2% octyl-POE.

**Preparation of *in vitro* folded OmpF.** OmpF inclusion bodies were expressed and folded as described previously (17) with slight modification. The purified OmpF from inclusion bodies was folded by a 20x dilution in 50 mM Tris-HCl, pH 8.0, 1 mM DTT (dithiothreitol) and 0.1 mM EDTA containing a mixture of 1% (w/v) DDG (n-dodecyl-β-D-glucopyranoside) and 0.4% (w/v) DDM (n-dodecyl-β-D-maltoside). After a stationary incubation at 37°C for at least 3 days, the sample was precipitated by mixing 1:9 with

cold ethanol (see above) and then the folded trimeric OmpF was recovered by resuspending in 20 mM Tris-HCl, pH 7.4, 0.5% (v/v) Octyl POE. To completely exchange the folding detergents to another detergent, buffer exchange was achieved by anion exchange chromatography using a 1-ml HiTrap Q Sepharose column equilibrated with 20 mM Tris-HCl, pH 7.4, 0.5% (v/v) octyl POE. The refolded OmpF was eluted using a linear salt gradient.

**Preparation of LPS.** Samples of LPS, Smooth (*E. coli* 055:B5), Ra (from *E. coli* EH-100), Rc (*E. coli* J5), Rd (*E. coli* F-583; Rd2) and Re (*S. minnesota* strain Re595) were from Sigma-Aldrich (Dorset, UK). Each was dissolved in 20 mM Tris-HCl, pH 7.4 to give a final concentration of 2 mg/ml. The LPS solution was sonicated in a water bath for 20 min and temperature-cycled 6 times between 4 and 70°C. The resulting solution was kept at 4°C overnight before use.

**Dynamic light scattering.** DLS measurements were performed with Zetasizer Nano (Malvern instrument Ltd.). 50 µl of protein at 0.1-0.5 mg/ml in a 45 µl quartz glass cuvette (Hellma 105.251-QS) was measured at 25°C in triplicates. Size measurements and data analysis were performed by Zetasizer software.

**X-ray crystal structure determination of *Enterobacter cloacae* OmpE36.** Genomic DNA of *Enterobacter cloacae* was obtained from Basilea Pharmaceutica (Basel, Switzerland). The *ompE36* gene including the signal sequence was amplified using PCR, digested with *NcoI* and *XbaI* and ligated with the arabinose-inducible vector pBAD24 containing an ampicillin resistance gene. The ligated product was electroporated into DH5α competent cells and plated on LB-ampicillin plates (100 µg/ml) for overnight incubation at 37°C. Screening for positive clones was done using colony PCR and clones were confirmed by DNA sequencing (MWG Eurofins). Porin-deficient *E. coli* omp8 competent cells ( $\Delta ompA \Delta ompC \Delta ompF \Delta lamB$ ) (53) were transformed with one of the positive clones and protein was expressed using 0.1% arabinose for induction (37 °C, 3 h). The cells were harvested by centrifugation and lysed with a cell disrupter (0.75 kW; Constant Systems; 1 pass at 23 kPSI). Total membranes were collected by ultracentrifugation using a 45 Ti rotor (Beckman; 45 mins; 42,000 rpm). The resulting membrane pellet was extracted twice with 0.5% N-

Lauroylsarcosine (sarkosyl) detergent (in 20 mM HEPES, pH 7.5) followed by centrifugation to solubilize and remove inner membrane proteins, followed by an overnight extraction at 4°C in 1% lauryldimethylamine-oxide (LDAO) in 10 mM HEPES, 50 mM NaCl, pH 7.5 to solubilize the OMPs. LDAO extraction was followed by ultracentrifugation (30 mins, 42,000 rpm) and the supernatant was subjected to Resource-Q anion exchange chromatography in 0.2% LDAO at pH 7.5 (10 mM Hepes, 50 mM NaCl). After elution, the protein was further purified by gel filtration chromatography in 10 mM HEPES, 100 mM LiCl, 0.4% C8E4, pH 7.5. The purified protein was concentrated to 24 mg/ml. Crystal trays were set up using 10 mg/ml and 15 mg/ml concentrations and the crystal hits obtained in each case were optimized to obtain good quality crystals. The optimized crystal condition for OmpE36 was 0.4 M ammonium sulfate, 0.1 M MES, 10% (w/v) PEG 3350, pH 6.5. Crystals were harvested, cryoprotected with glycerol (~20%) and flash-frozen in liquid nitrogen. X-ray diffraction data were collected at the Diamond Light Source (Didcot, UK) and processed using XDS (54). The crystal structure was solved using data to 1.45 Å resolution by molecular replacement using Molrep (55) with *E. coli* OmpC (PDB code 2J1N) as the search model. The refinement was done in Refmac5 (55) and COOT (56) was used for model (re)building. The data collection and refinement statistics are summarized in Table S1.

### **Small angle neutron scattering (SANS) sample preparation and data collection.**

LPS was removed from *in vivo* folded d-OmpF using size exclusion chromatography on a Superose 12 column equilibrated with 20 mM Tris, pH 7.5, 300 mM NaCl, 10 mM EDTA and 0.5% octyl-POE. The purified d-OmpF was then used to form complexes by incubation with Ra-LPS at a 1:5 dOmpF/LPS molar ratio at 37°C overnight. To remove unbound LPS, the samples were again passed through the same Superose 12 column using the same buffer without EDTA. After SEC, the proteins in the fractions were precipitated by pre-chilled ethanol at 1:9 ratio (90% ethanol in total). The protein pellet was resuspended with 50 mM potassium phosphate, pH 7.5, 150 mM NaCl containing 0.5% h/d SDS in 13%, 27%, 41%, 77% and 100% D<sub>2</sub>O and dialyzed against the same buffer. 50 mM sodium phosphate buffer, pH 7.4, 300 mM NaCl was made at two different H<sub>2</sub>O/D<sub>2</sub>O ratios (13 % (v/v) and 100 % (v/v) D<sub>2</sub>O). Hydrogenous SDS (h-SDS) was added to 13% D<sub>2</sub>O buffer (which has the same scattering length density (SLD) as h-SDS and is thus the lowest SLD we can use) to give a final concentration

of 0.5 % (w/v) while 5% (w/v) h-SDS and 95% (w/v) deuterated SDS were added to 100% D<sub>2</sub>O buffer to give a final concentration of 0.5 % (w/v) h/d-SDS with the same SLD as 100% D<sub>2</sub>O. These two buffers were used in SANS and as stock solutions to prepare 27%, 41% and 77% D<sub>2</sub>O buffer in which the SLD of SDS was also matched to the solvent.

SANS experiments were conducted on the D22 beamline at the ILL, Grenoble, France at 20°C using 1 mm quartz rectangular cuvettes. The data were collected using the following instrument configuration: sample detector distances were 11.2, 5.6 and 1.5 m and the collimation lengths were 11.2, 5.5 and 2.8 m, respectively. This configuration covers the momentum transfer ( $Q = 4\pi\sin\theta/\lambda$ , where  $2\theta$  is the scattering angle and  $\lambda$  is the neutron wavelength, in this case = 6 Å) range between 0.01 and 0.35 Å<sup>-1</sup>. The data reduction was carried out using standard protocols implemented in GRASansP software (<https://www.ill.eu/instruments-support/instruments-groups/groups/lss/grasp/home/>). Neutron scattering contrasts for D/H OmpF-LPS complexes were calculated using program SASSIE (<https://sassie-web.chem.utk.edu/sassie2/>) (57). Experimental scattering parameters of D/H OmpF-LPS complexes were determined using Bayesian approach for indirect Fourier transformation of scattering curves obtained in reciprocal space ([www.http://www.bayesapp.org/](http://www.bayesapp.org/)) (58, 59). The match points of both protein and LPS were determined as  $\sqrt{I_0}$  vs D<sub>2</sub>O fraction in solution ( $I_0$  normalized to the sample concentration). A Stuhrmann plot of the square of the radius of gyration ( $R_g^2$ ) versus the inverse of the contrast between the scattering object and the solvent at different D<sub>2</sub>O concentrations ( $\Delta\rho$ ) (33) was obtained using the routine implemented in the program MULCh <http://smb-research.smb.usyd.edu.au/NCVWeb/> (60).

**Small-angle X-ray scattering (SAXS).** SAXS data for dodecylmaltoside solubilized OmpF-LPS complexes were collected at 20°C on the beamline BM29 (61) at (ESRF, France). Scattering curves were recorded at a wavelength of 1.008 Å at a sample-detector distance of 2.85 m covering the momentum transfer range  $0.05 < Q < 0.45 \text{ Å}^{-1}$ . The sample was gel-filtered prior to SAXS and the final concentration of samples was between 0.4 and 1 mg/ml. Initial data processing and averaging were carried out according to (62). The scattering curves were converted to the real space pair-

distribution function  $P(r)$  using a Bayesian approach ([www. http://www.bayesapp.org/](http://www.bayesapp.org/)) (58, 59) The detergent shell of solubilized *in vitro* folded OmpF and OmpF-LPS complexes was modelled using the program MEMPROT (30).

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## Protein Data Bank accession number

The coordinates and structure factors for OmpE36-LPS complex have been deposited in the Protein Data Bank in Europe (PDBe) with accession code 5FVN.

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## Figure Legends

**Figure 1. Binding of LPS to OmpF causes slower mobility of complexes on SDS-PAGE.** (A) Structure of LPS from *E. coli* with the R3 core structure, including that of the Rc LPS from *E. coli* J5 used in this study (63) (non-stoichiometric additions are shown with dotted lines). In Lipid A, GlcN<sub>I</sub> and GlcN<sub>II</sub> are, respectively, the reducing and non-reducing glucosamine residues. The inner core usually comprises 2 or 3 Kdo (3-deoxy-D-manno-oct-2-ulosonic acid) and 3 Hep (L-glycero-D-manno-heptose) molecules. This region is phosphorylated at several sites. The variable tri-hexose backbone forms the outer core with varying side chains, shown here as present in the R3 form are Glucose (Glc), N-Acetylglucosamine (GlcNac) and Galactose (Gal). These link to the long O-antigen polysaccharide (O-PS) region found only in smooth strains. The depiction of the rough Ra to Re mutants which define the different chemotypes is based upon the original classification in *Salmonella minnesota* (64). The Rd LPS used here is from an Rd2 mutant, as shown. (B) Characteristic ladder, on 10% SDS-PAGE, of OmpF resulting from LPS binding. *In vitro* folded LPS-free OmpF (left) and an identical sample mixed with a 5-fold molar excess of Ra-LPS (right). (C) Samples of *in vitro* folded OmpF+Ra LPS as in (B) mixed with 5 mM MgCl<sub>2</sub>, CaCl<sub>2</sub>, or EDTA. (D) *In vitro* folded OmpF mixed with 5-fold molar ratio of LPS variants of increasing size. Sm= Smooth LPS. (E) *In vitro* folded OmpF mixed with increasing molar ratios of Ra-LPS.

**Figure 2. Removing positively charged residues decreases the amount of LPS bound to OmpF** (A) Localization of positively charged residues on the extracellular side of OmpF trimer (PDB code: 2OMF). We divided these residues into group A in the cleft (magenta) and group B at the perimeter (yellow) separated by the red dashed line. This and other structural images were created using PyMol (65). (B) Native OmpF, WT and site A mutant proteins, purified from the OM with bound LPS, analyzed

on 10% SDS-PAGE gel stained with Coomassie Blue. The double and triple glutamine mutations retain a tail of LPS-bound forms which contrasts to the quadruple glutamate mutations (see Fig. 3F).

**Figure 3. LPS binding site B is essential for OmpF trimerisation *in vivo* but not *in vitro*.** The abbreviations describe the mutations applied at each site; e.g. A-Gln = all basic residues in site A replaced by glutamine or AB-Glu = all basic residues in sites A and B replaced by glutamate. (A) Purification of A-Gln mutant protein. MM- molecular weight markers, P; membrane pellet, S; Supernatant, W1 and W2 supernatants after washes 1 and 2, BEX and EX; boiled and native OmpF extraction samples showing monomers and trimers respectively, BOF and NOF; boiled and native WT OmpF control samples from *in vitro* folded stock. T = position of trimer band on gel, M = position of monomer band on gel. (B) Purification of B-Gln mutant protein. Pre I; whole cell pellet before induction, Post I; whole cell pellet after 1h induction showing OmpF band at 37 kDa, Sol; supernatant after cell breakage, BW1 and W1; boiled and native supernatants from wash 1, BEX and EX as above showing lack of trimeric OmpF extracted with SDS and NaCl. (C) Summary table of results for mutants. Expression indicates band observed at 37 kDa after induction, trimerization indicates that intact trimers were purified from the OM. (D) Solubilization of A-Glu, B-Glu and AB-Glu inclusion bodies in urea, showing different migration of unfolded monomers on SDS-PAGE. (E) *In vitro* folding of trimeric porins. RWT; *in vitro* folded WT, NWT; native WT purified from OM. RA, RB, RAB; *in vitro* folded A-Glu, B-Glu and AB-Glu mutants with and without boiling. A-Glu and B-Glu fold fully *in vitro* but no heat-modifiable trimer is evident in AB-Glu samples. T and M are defined as in (A). (F) LPS binding to *in vitro* folded mutants. WT, A-Glu (Site B intact), B-Glu (Site A intact) without added calcium, plus 5 mM CaCl<sub>2</sub> and 5 mM EDTA. Note the clear effect of calcium removal on the A-Glu mutant, indicating a role for calcium ion(s) in site B.

**Figure 4. SANS data indicates that LPS binds at the periphery of OmpF in SDS solution.** (A) Scattering data for deuterated (d-)OmpF in complex with hydrogenous Ra-LPS after size exclusion chromatography. Q = momentum transfer which is a product of the scattering angle and neutron wavelength (6 Å) (see methods). Fitted lines were generated by the program BayesApp to calculate the P(r) vs distance (r) plots (see Table 1 for fitting parameters and Figure S8 for an enlarged Fig. 4A). P(r)

is the real space pair distance function which describes the distribution of pairs of scattering centers within the complex (see methods). (B)  $P(r)$  plot calculated from data in (A) using the same color scheme shows that at 13%, 27% and 41%  $D_2O$ , where LPS scattering is minimal, the plots resemble free OmpF (32). At 77%  $D_2O$ , when the d-OmpF scattering is minimal, the plot describes small groups of scattering centers separated by about 90 Å. This corresponds to groups of LPS arranged at fixed sites around the trimer as in the case of OmpF-colicin complexes (32). (C) The line of best fit to the Stuhrmann Plot, the square of the radius of gyration ( $R_g^2$ ) versus the inverse of the contrast ( $1/\Delta\rho$ ) (see methods), has a negative value of  $\alpha$  (see results), a result most easily appreciated from the apex of the parabola being at negative values of  $1/\Delta\rho$ . This indicates that the low nSLD LPS has a larger radius of gyration ( $R_g$ ) than the high nSLD OmpF and is likely to be situated at the periphery of the complex.

**Figure 5. X-ray crystal structure of the *E. cloacae* OmpE36-LPS complex.** (A) Cartoon view of the OmpE36 trimer from the top with the bound LPS molecules indicated by stick models. The bound calcium ion is shown as a green sphere. (B) Side view showing LPS A and LPS B binding. The approximate boundaries of the hydrophobic core of the OM are indicated by horizontal lines. (C) Close-up of LPS B with polar interactions between the LPS and OmpE36 shown by dashed lines. (D) Close-up of the boxed region in (B), showing the interactions of the central Arg213 with both LPS A and LPS B. For clarity, some LPS B acyl chains have been removed. (E) View from the extracellular side highlighting interactions between LPS B and OmpE36. (F) Close-up of the calcium ion bridging LPS B and OmpE36.

**Figure 6. 2D map showing interactions between OmpE36 residues and LPS A and B.** The figure was generated using Poseview (66). GlcN<sub>I</sub> and GlcN<sub>II</sub> are the reducing and non-reducing 2-amino-2-deoxy- $\alpha$ -D-glucopyranose residues of lipid A (glucosamine) respectively, Kdo denotes 3-deoxy-D-manno-oct-2-ulosonic acid and GMH corresponds to L-glycero-D-manno-heptopyranose. Acyl chains of lipid A are shown by \*.

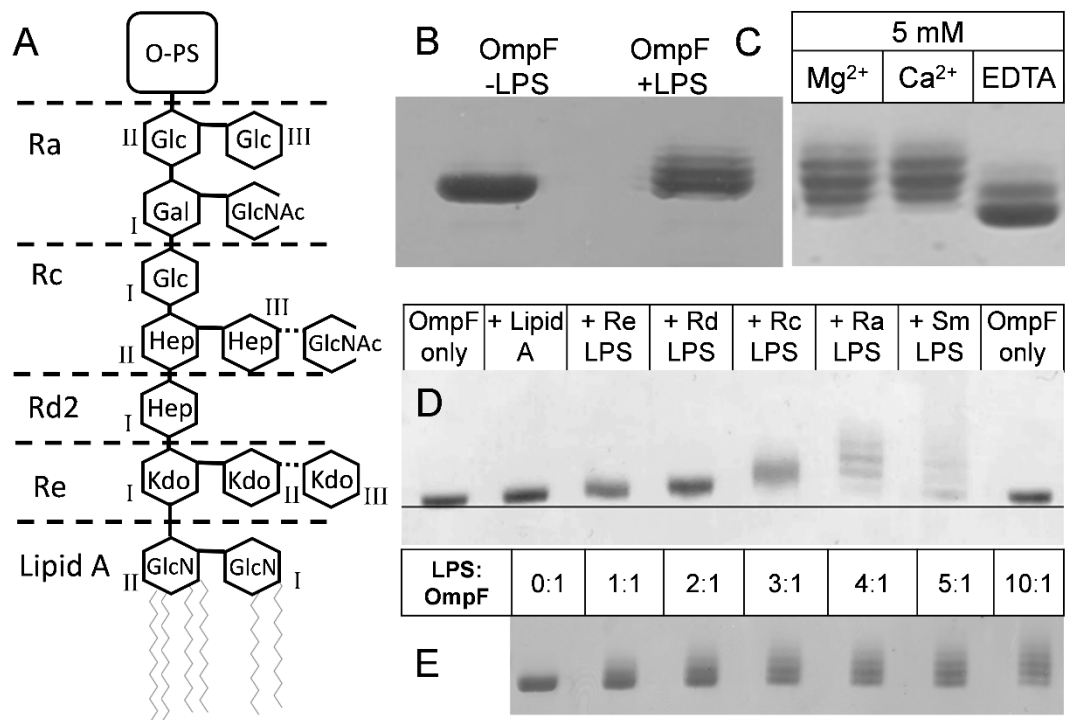


Figure 1

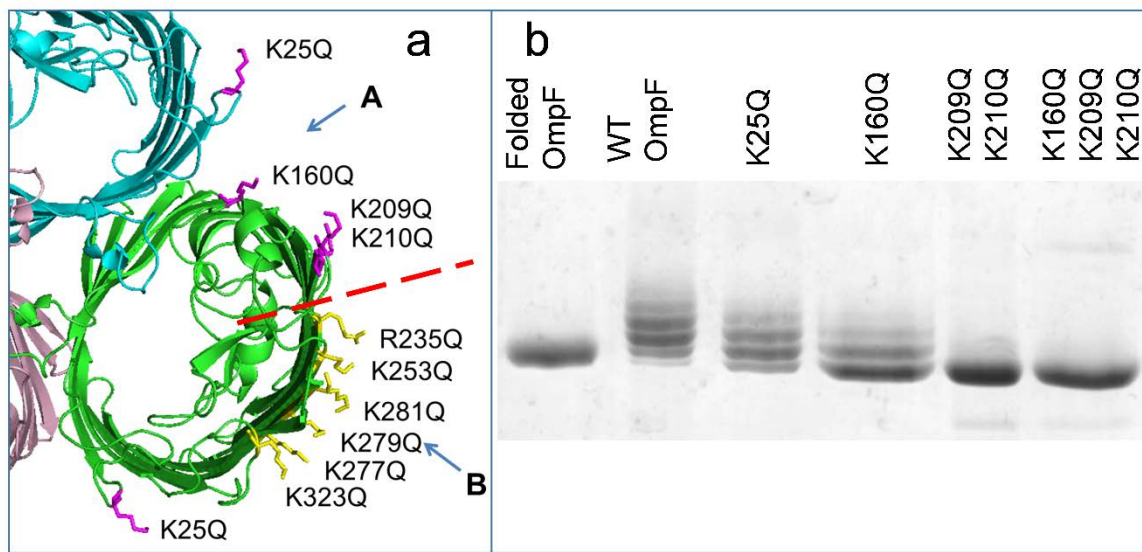


Figure 2

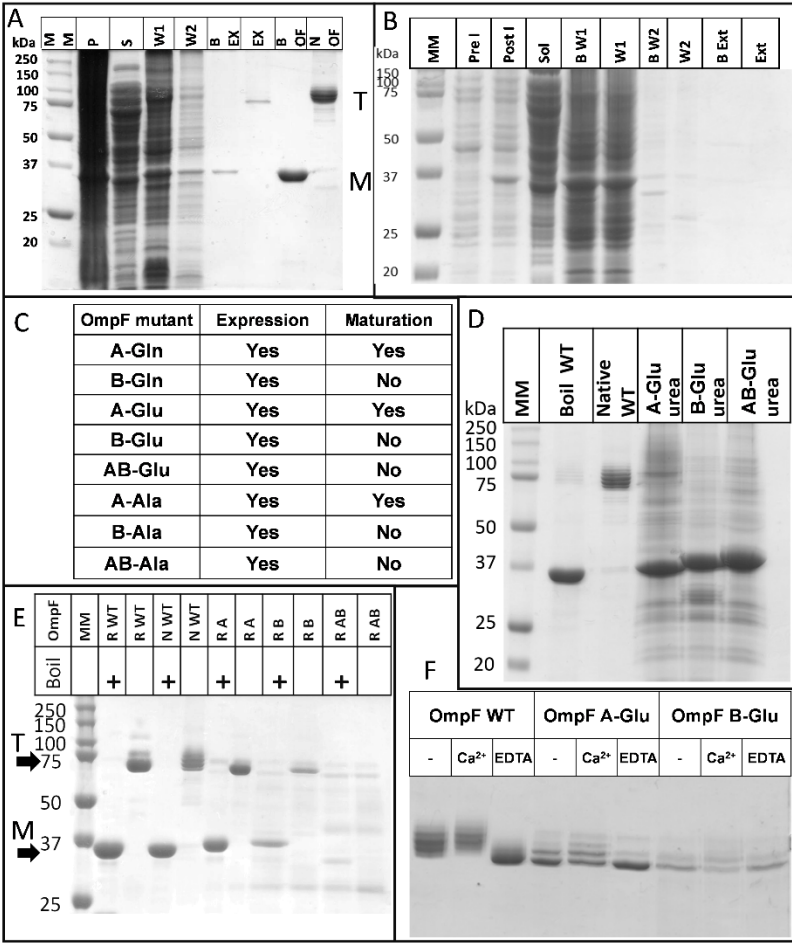


Figure 3



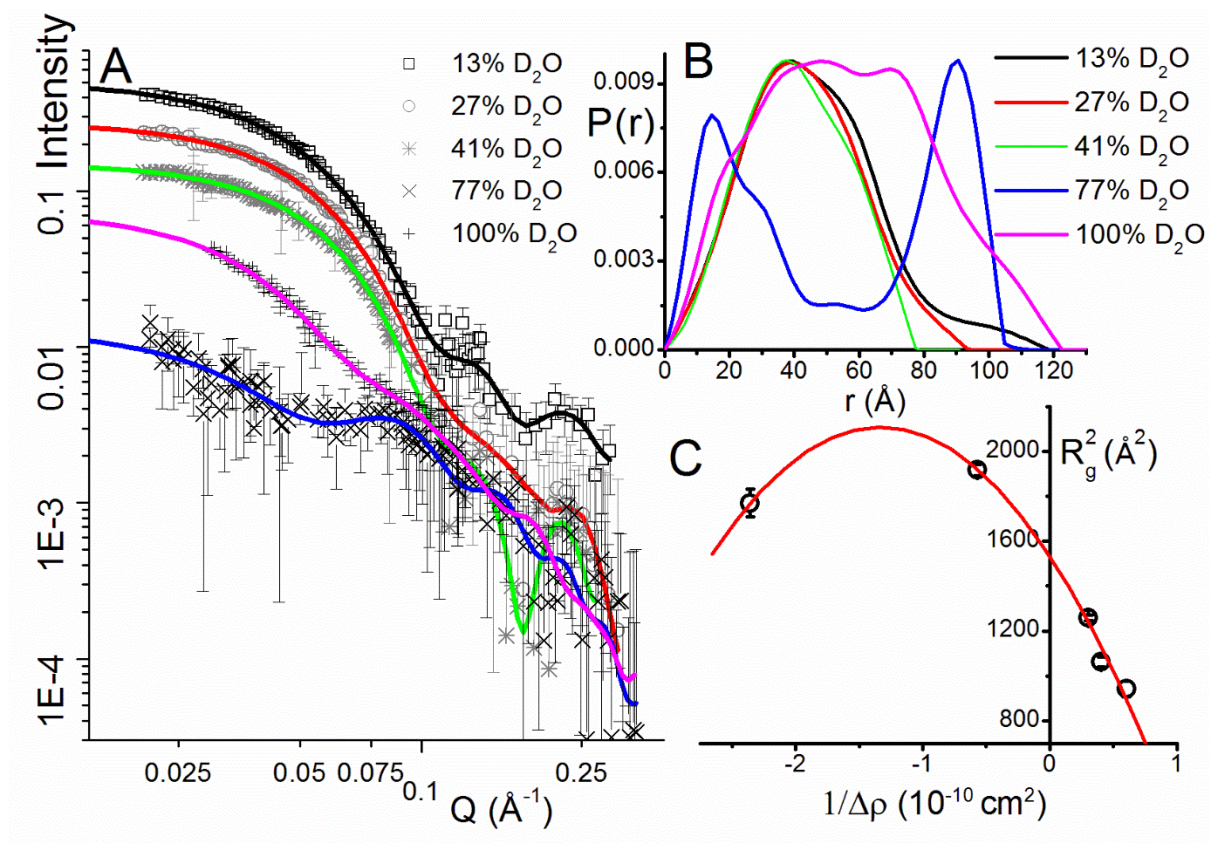


Figure 4

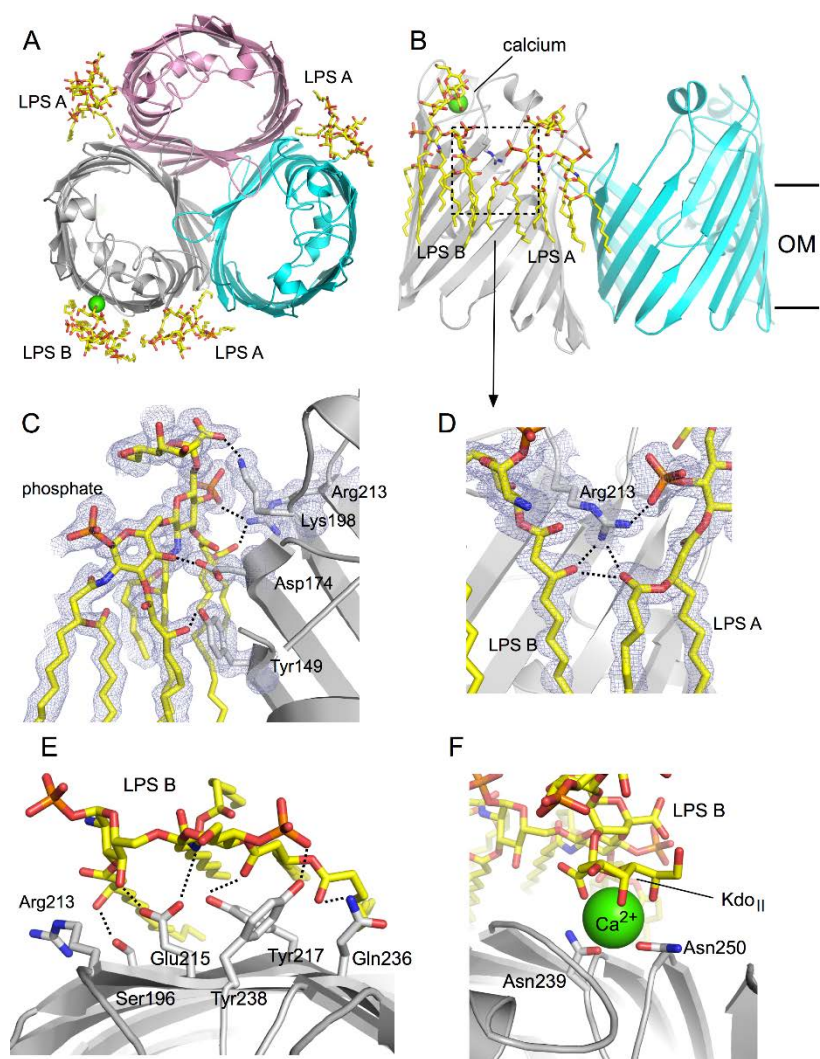


Figure 5

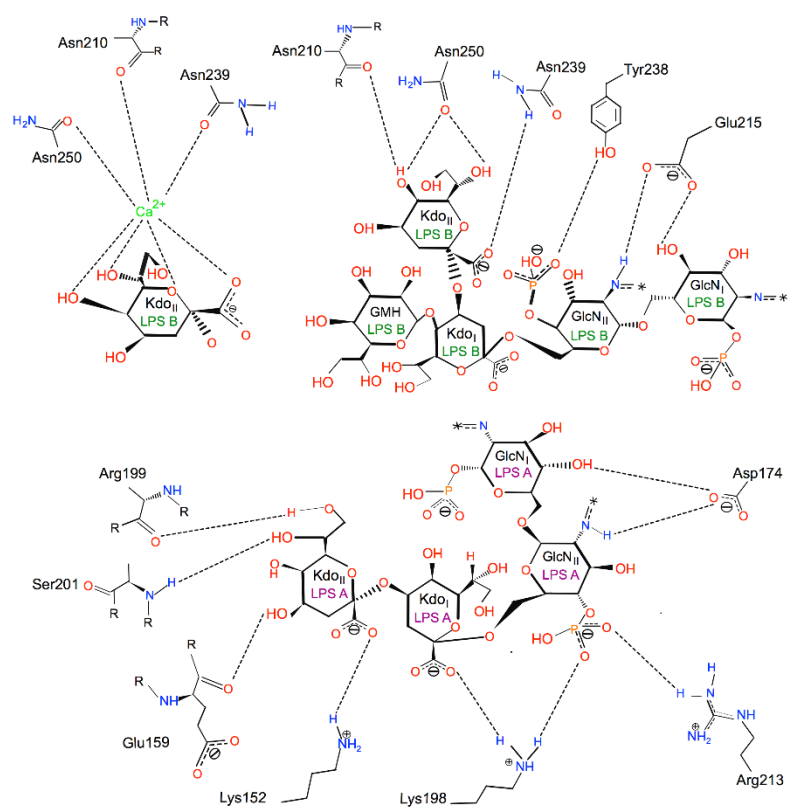


Figure 6

Supplementary Appendix for

**Gram-negative trimeric porins have specific LPS binding sites that are essential for porin biogenesis.**

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†These two authors contributed equally to the paper.

\*Corresponding authors

**Table S1** (A) Data collection and refinement statistics for OmpE36

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**Data collection**

Beamline	DLS i02
Wavelength	0.9796
Space group	P 2 <sub>1</sub>
Cell dimensions (a,b,c)	109.75 123.26 116.01
( $\alpha,\beta,\gamma$ )	90.00 91.01 90.00
Molecules/AU	6
Solvent content	65
Resolution (Å)	48.92-1.45
Completeness	99.6 (100)
Redundancy	3.7 (3.7)
Rmerge (%)	8.4 (78)
Rpim (%)	4.9 (46)
CC (1/2)	0.98 (0.62)

**Refinement**

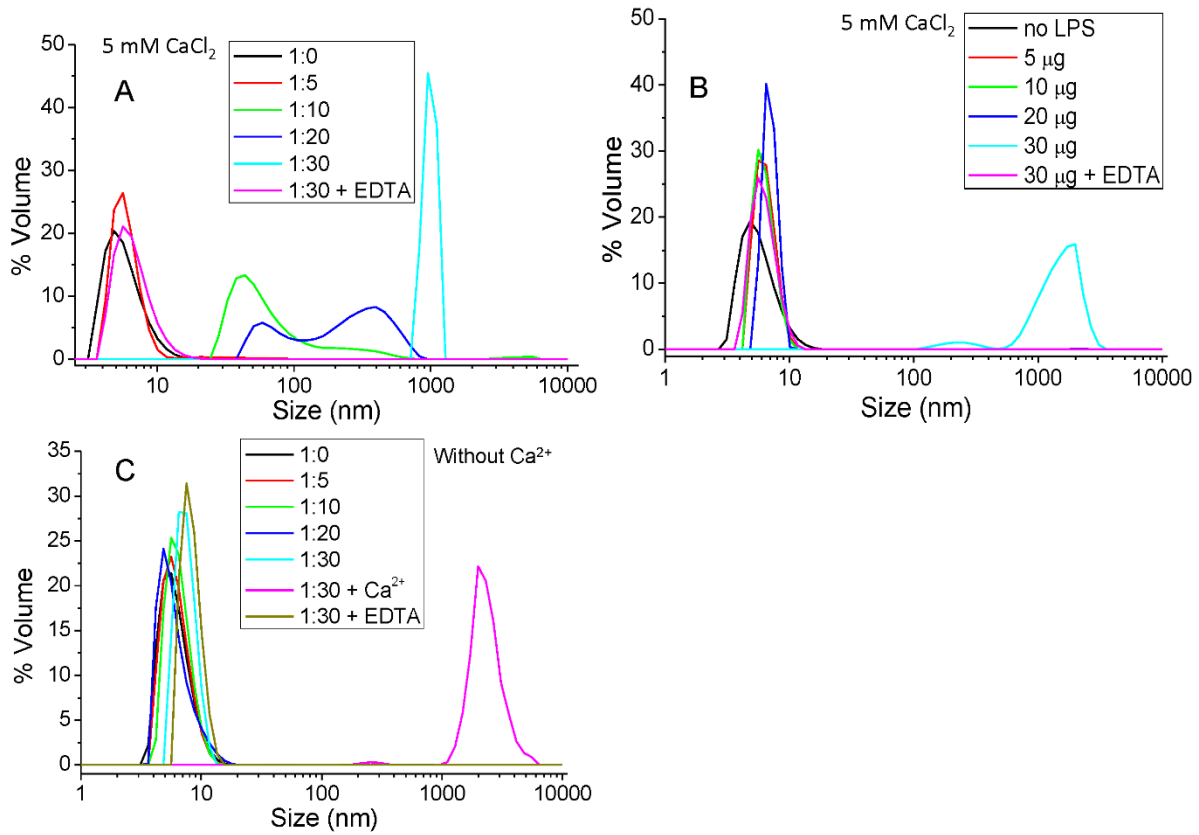
Resolution (Å)	48.92-1.45
Reflections (n)	529555
R <sub>work</sub> /R <sub>free</sub> (%) <sup>*</sup>	15.2/17.8
Atoms (n)	
Protein/solvent	16245/1910
ligand/detergent	1239/231
B factors (Å <sup>2</sup> )	
Protein/solvent	17/30
ligand/detergent	37/42
Rmsd	
bond lengths (Å)	0.0095
bond angles (°)	1.5091
Ramachandran plot (%)	
Most favoured/disallowed	95.5/0.6
Molprobit clashscore	1.37

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Values in parentheses refer to the highest resolution shell<sup>\*</sup>R<sub>free</sub> was computed as for R<sub>work</sub> using a test set (5%) of randomly selected reflections that were omitted from the refinement

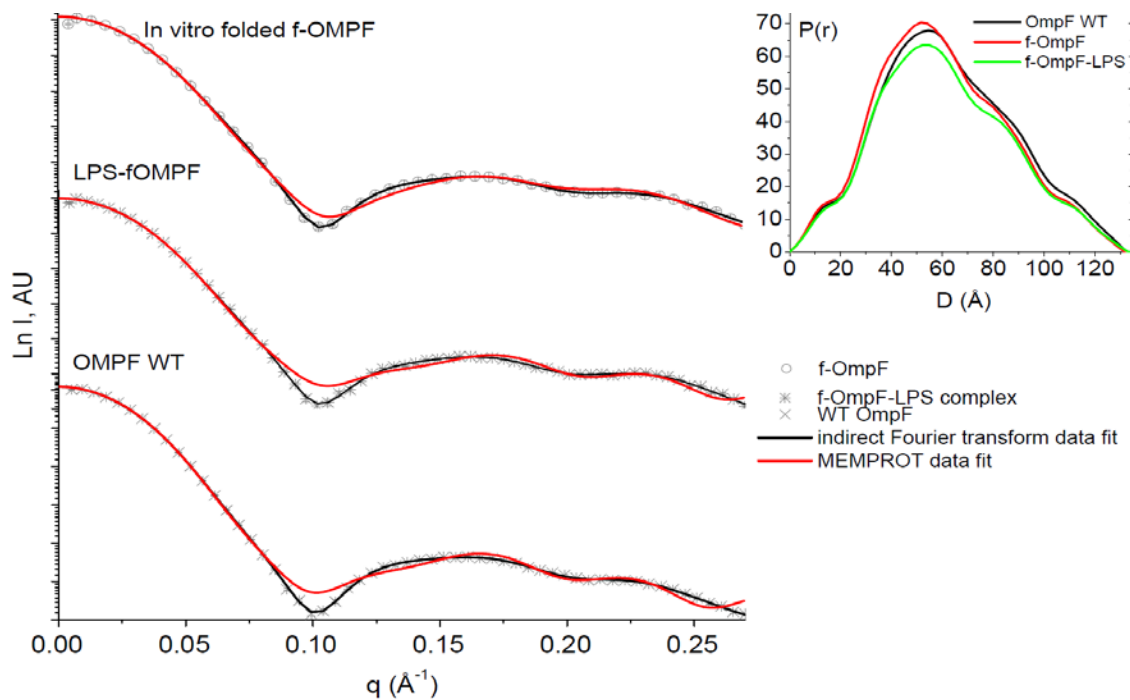
**Table S1 (B)** Interaction distances (in Å) measured for the OmpE36-LPS complex.

Residue of E36-C chain	LPS atom (moiety)	H-bond distance (2.3-3.6 Å)	Residue/LPS molecule	Metal ion	Ionic interaction distance (Å)
174 Asp, OH-	2 PA1, O4 (LPS-A)	2.63	250, Asn, O-	Calcium	2.36
174 Asp, OH-	3 GCS, N2 (LPS-A)	2.82	239, Asn, O-	Calcium	2.49
149 Tyr, OH-	7 FTT, O3 (LPS-A)	2.69	210, Asn, O-	Calcium	2.35
213 Arg, NH <sub>2</sub> -	11 MYR, O1 (LPS-A)	3.56	5, KDO, O7- (LPSB)	Calcium	2.59
213 Arg, NH <sub>2</sub> -	9 FTT, O2 (LPS-A)	3.6	5, KDO, O1- (LPSB)	Calcium	2.46
213 Arg, NH <sub>2</sub> -	6 PO <sub>4</sub> , O3 (LPS-A)	2.78	5, KDO, O5- (LPSB)	Calcium	2.45
198 Lys, NH <sub>2</sub> -	4 KDO, O1 (LPS-A)	2.84	5, KDO, O6- (LPSB)	Calcium	2.59
198 Lys, NH <sub>2</sub> -	3 GCS, O4 (LPS-A)	3.6			
198 Lys, NH <sub>2</sub> -	6 PO <sub>4</sub> , O1 (LPS-A)	2.8			
160 Asp, OH-	5 KDO, O5 (LPS-A)	2.52			
199 Arg, O-	5 KDO, O8 (LPS-A)	2.86	<b>28 hydrogen bonds between LPS (A or B) and OmpE36</b>		
159 Glu, O-	5 KDO, O4 (LPS-A)	2.8			
152 Lys, NH <sub>2</sub>	5 KDO, O1 (LPS-A)	2.91	<b>1 hydrogen bond between LPS A and LPS B</b>		
152 Lys, NH <sub>2</sub>	5 KDO, O6 (LPS-A)	3.2			
152 Lys, NH <sub>2</sub>	5 KDO, O5 (LPS-A)	2.81	<b>7 ionic interactions between calcium and LPS/OmpE36</b>		
201 Ser, NH-	5 KDO, O7 (LPS-A)	2.99			
<b>11 MYR, O1 (LPSA)</b>	<b>7 FTT, O3 (LPS-B)</b>	<b>3.09</b>			
213, Arg, NH-	7 FTT, O3 (LPS-B)	3.58	<b>Abbreviations</b>		
196, Ser, OG	7 FTT, O3(LPS-B)	2.9	2-amino-2deoxy-β-D-glucopyranose	GCS	
215, Glu, OH-	9 FTT, O2 (LPS-B)	3.23	2-amino-2deoxy-α-D-glucopyranose	PA1	
215, Glu, OH-	2 PA1, O4 (LPS-B)	2.41	3-deoxy-alpha-D-manno-oct-2-ulosonic acid	Kdo	
250, Asn, OH-	5 KDO, O5 (LPS-B)	3.11	3-hydroxytetradecanoic acid	FTT	
217, Tyr, OH-	9 FTT, O2 (LPS-B)	3.54	Dodecanoic acid	DAO	
239, Asn, NH-	5 KDO, O1A (LPS-B)	2.79	Tetradecanoic acid	Myr	
210, Asn, OH-	5 KDO, O5 (LPS-B)	2.94	L-glycero-alpha-D-manno-heptopyranose	GMH	
215, Glu, OH-	3 GCS, N2 (LPS-B)	3.14			
236, Gln, NH-	12 DAO, O1 (LPS-B)	3.31			
238, Tyr, OH-	6 PO <sub>4</sub> , O3 (LPS-B)	2.64			
238, Tyr, OH-	3 GCS, O4 (LPS-B)	3.58			
251, Lys, NH-	6 PO <sub>4</sub> , O3 (LPS-B)	3.43			



**Figure S1** Dynamic light scattering measurements of the effect of LPS-OMPf ratio and divalent cations on the formation of OmpF-LPS aggregates. (A) Size distribution of OmpF-Ra-LPS complexes studied by dynamic light scattering in 5 mM  $\text{CaCl}_2$ , 1% SDS. (B) Increasing concentrations of Ra-LPS without OmpF in 5 mM  $\text{CaCl}_2$ , 1% SDS. The signal in the zero LPS sample is from SDS micelles. In each case 10 mM EDTA was added to the 1:30 ratio sample which removed all calcium ions and returned the complex size to a similar value before calcium addition. (C) OmpF-Ra-LPS complexes in 1% SDS without added calcium except 1:30 when 5 mM  $\text{CaCl}_2$  was added. This was followed by 10 mM EDTA to remove free  $\text{Ca}^{2+}$ .

## S2:A

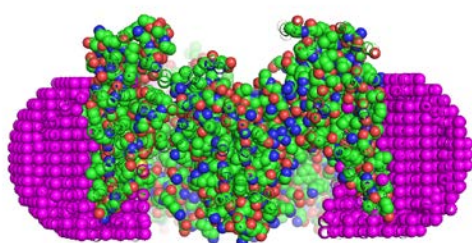
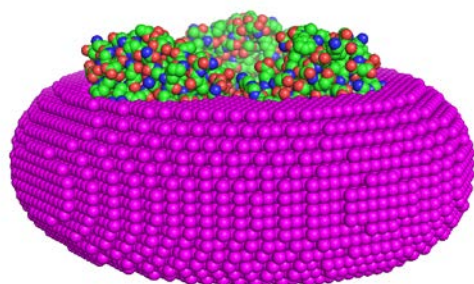


## S2:B

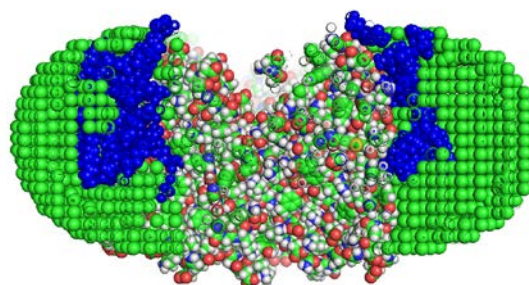
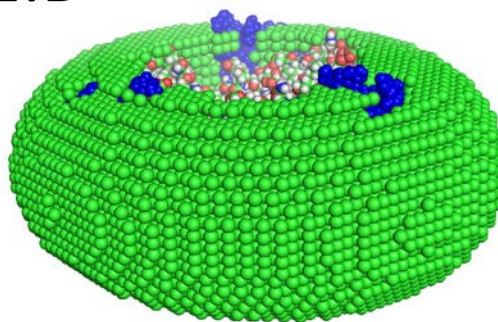
Sample	c, mg/ ml	Scattering parameters*			Modelling detergent shell**						
		$I_0$	$R_g$	$D_{\max}$	Protein (trans- membrane part) radius ( $\text{\AA}$ )	$R_g$ ( $\text{\AA}$ ) of the model	a, ( $\text{\AA}$ )	b, ( $\text{\AA}$ )	t, ( $\text{\AA}$ )	e	$\chi$
In vitro Refolded OmpF	0.42	441.63 $\pm 0.06$	46.2	128.9 $\pm 0.009$	35	45.9	35	40	5.0	1.13	1.53
In vitro Refolded OmpF- LPS complex	0.43	405.78 $\pm 0.06$	46.8	130.2 $\pm 0.008$	37	46.3	40	37	5.2	1.08	1.66
WT OmpF	0.65	439.67 $\pm 0.03$	47.6	129.8 $\pm 0.005$	37	47.6	40	39	5.3	1.08	1.97



S2:C



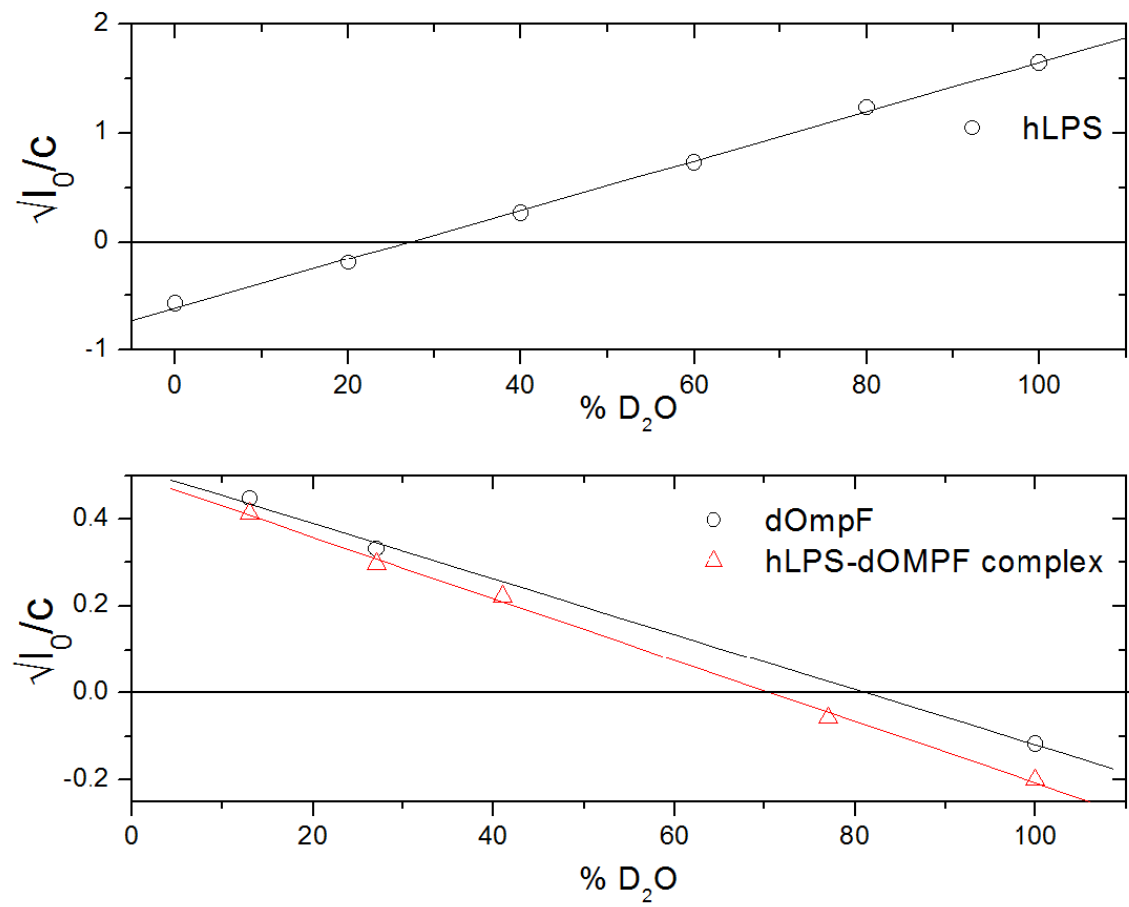
S2:D



**Figure S2** Small-angle X-ray scattering (SAXS). SAXS data for detergent solubilized OmpF-LPS complexes were collected on the beamline BM29 (1) at ESRF, France. (A) Scattering curves were recorded at a wavelength of 1.008 Å at a sample-detector distance of 2.85 m covering the momentum transfer range  $0.05 < q < 0.45 \text{ Å}^{-1}$ , where  $q = (4\pi\sin\theta)/\lambda$  and  $2\theta$  is the scattering angle. The sample was gel-filtered prior to the SAXS experiment, the concentration of samples was 0.4 and 1 mg/ml, the experimental temperature was 20°C. Initial data processing and averaging were carried out according to (2). The scattering curves were converted to the real space pair-distribution function  $P(r)$  using Bayesian approach ([www. http://www.bayesapp.org/](http://www.bayesapp.org/)) (3, 4). OmpF-WT = samples purified from the bacterial outer membrane with naturally associated LPS; f-OmpF = *in vitro* refolded OmpF (LPS-free); F-OmpF-LPS complex = *in vitro* refolded OmpF mixed with Ra-LPS. The detergent shell of solubilized *in vitro* folded OmpF and OmpF-LPS complexes was modelled using program MEMPROT (5) using data for DDM micelles from (6). (B) The hydrophobic hydrocarbon tails of the detergent that assemble around the protein surface are modelled using Memprot (5) as an elliptical hollow torus of height  $a$  and cross-sectional minor and major axes  $b/e$  and  $b \times e$ , where  $e$  is the

ellipticity of the torus. The hydrophilic region occupied by the detergent polar headgroups is modelled as an exterior shell of constant thickness  $t$  surrounding the inner hydrophobic torus (5). \* SAXS data were obtained after a gel-filtration step for OmpF solubilised in 0.2 %DDM. \*\*Detergent shell modelling used a 2LPS per OmpF monomer model. (C) Fitted model for *in vitro* folded OMPF in DDM (Magenta) using Memprot. (D) Fitted model for WT OMPF purified with bound LPS in DDM using Memprot. Two LPS molecules (BLUE) per trimer. Note how the detergent micelle thickness (green) is extended compared to the LPS free sample in (C).

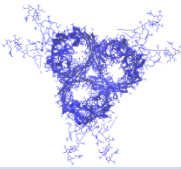
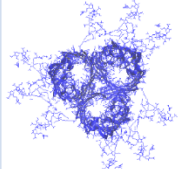
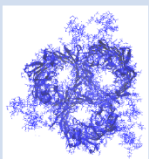
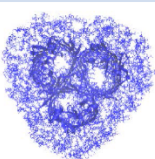
A)



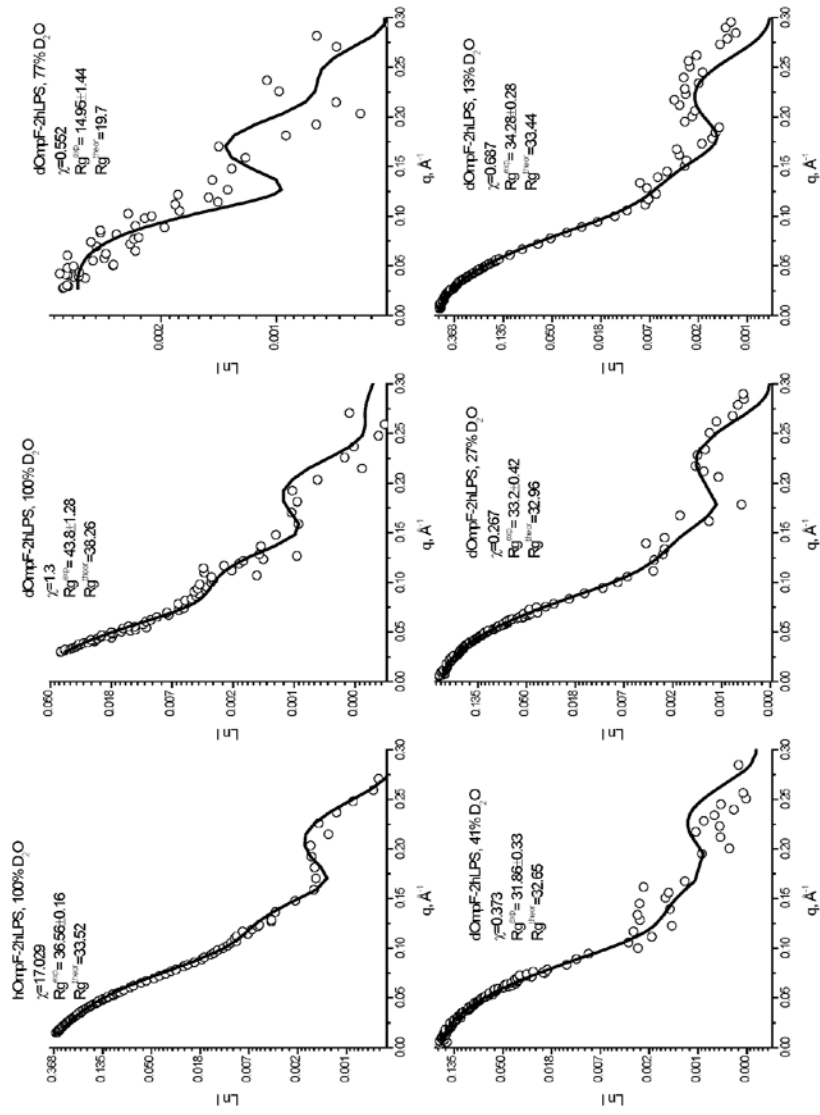
(B)

LPS/OmpF <sub>monomer</sub>	%D <sub>2</sub> O matchpoint
1:1	75.2
2:1	70.5
3:1	66.7
8:1	54.5
9:1	52.9

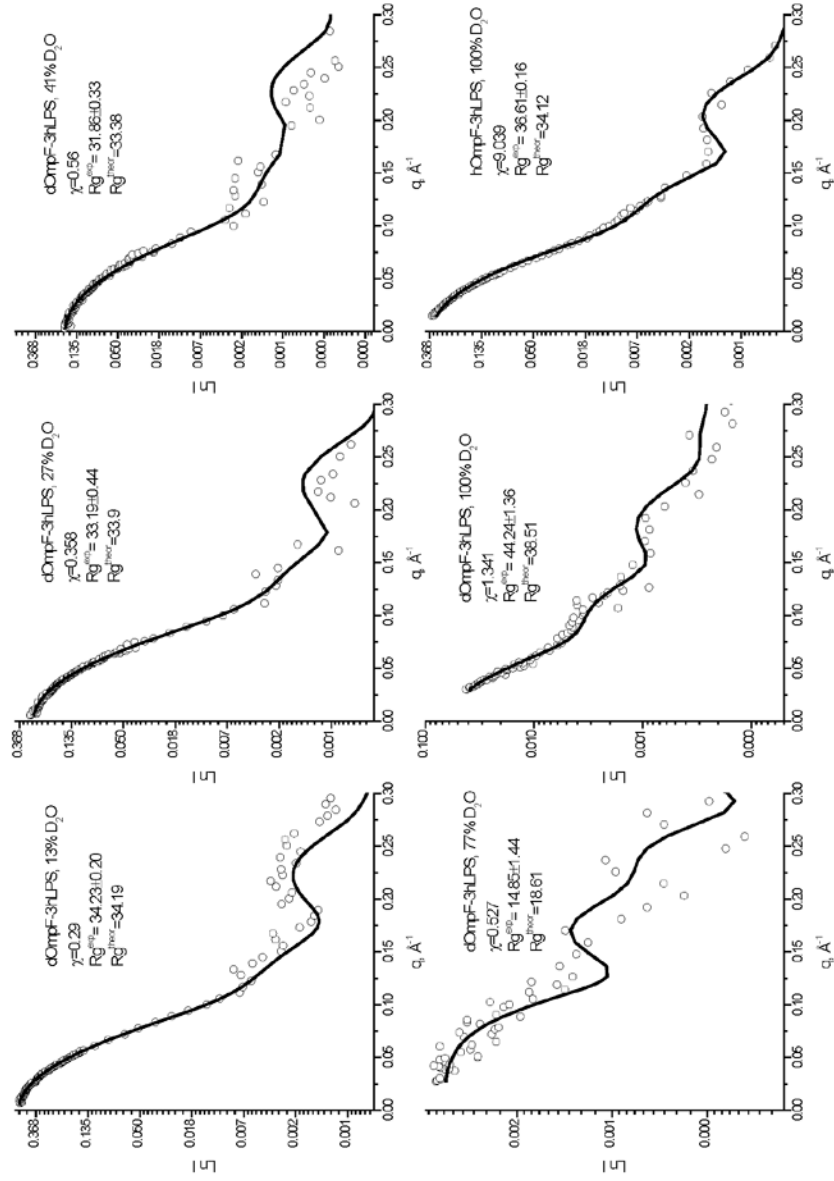
(C)

Contrast/Model	2LPS per monomer (extended)	3LPS per monomer (extended)	2LPS per monomer (compact)	8LPS per monomer
				
13% D <sub>2</sub> O (D/H)	0.687	0.29	0.878	3.478
27% D <sub>2</sub> O (D/H)	0.267	0.358	0.532	2.264
41% D <sub>2</sub> O (D/H)	0.373	0.56	0.726	2.042
77% D <sub>2</sub> O (D/H)	0.552	0.527	0.687	0.658
100% D <sub>2</sub> O (D/H)	1.3	1.341	1.731	1.043

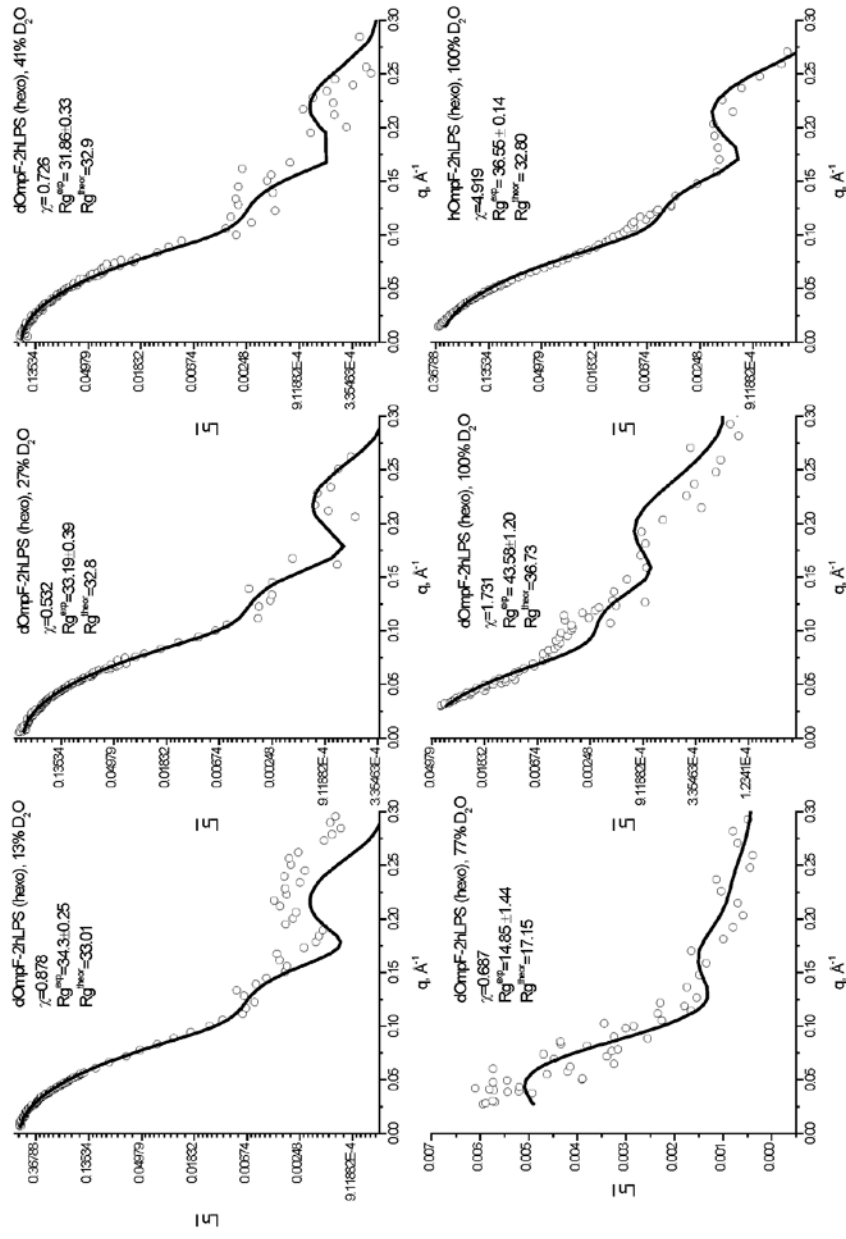
# (D) 2LPS extended model



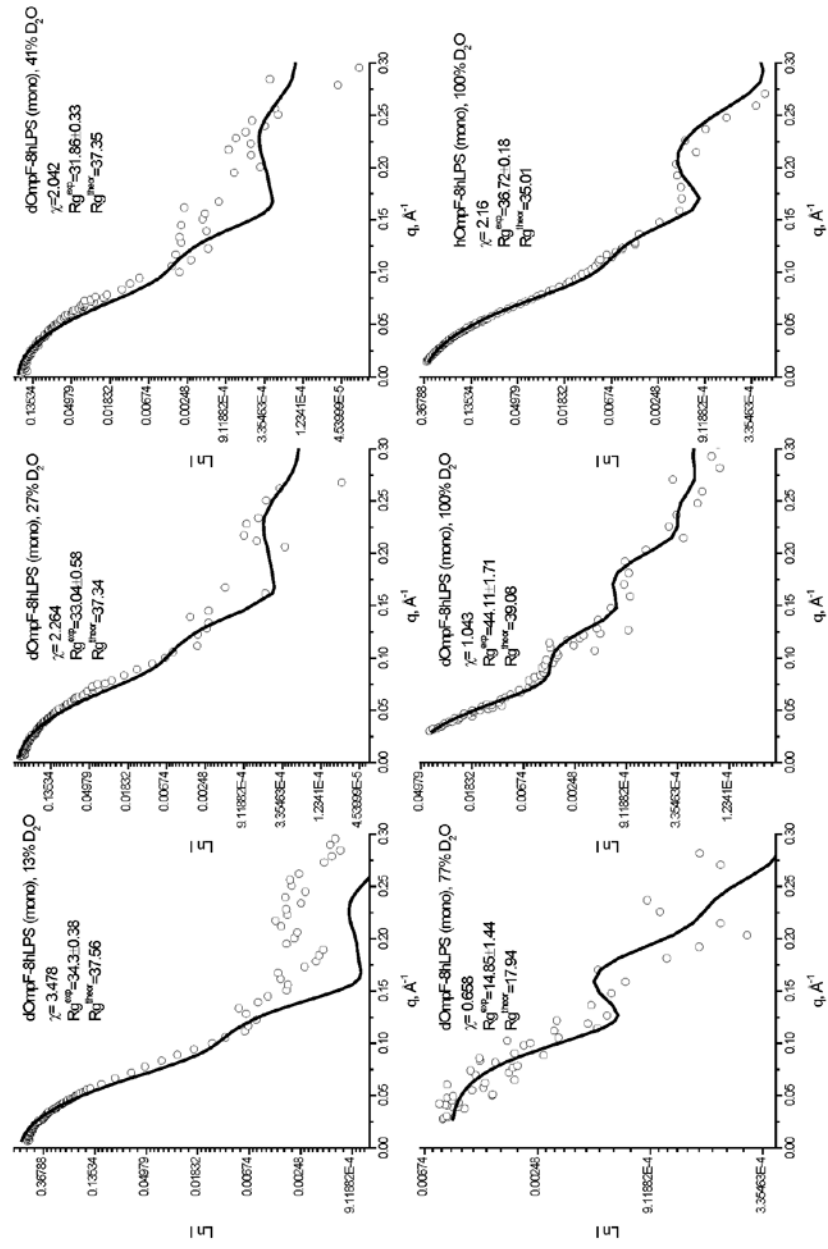
# (D) 3LPS extended model



## (D) 2LPS compact model

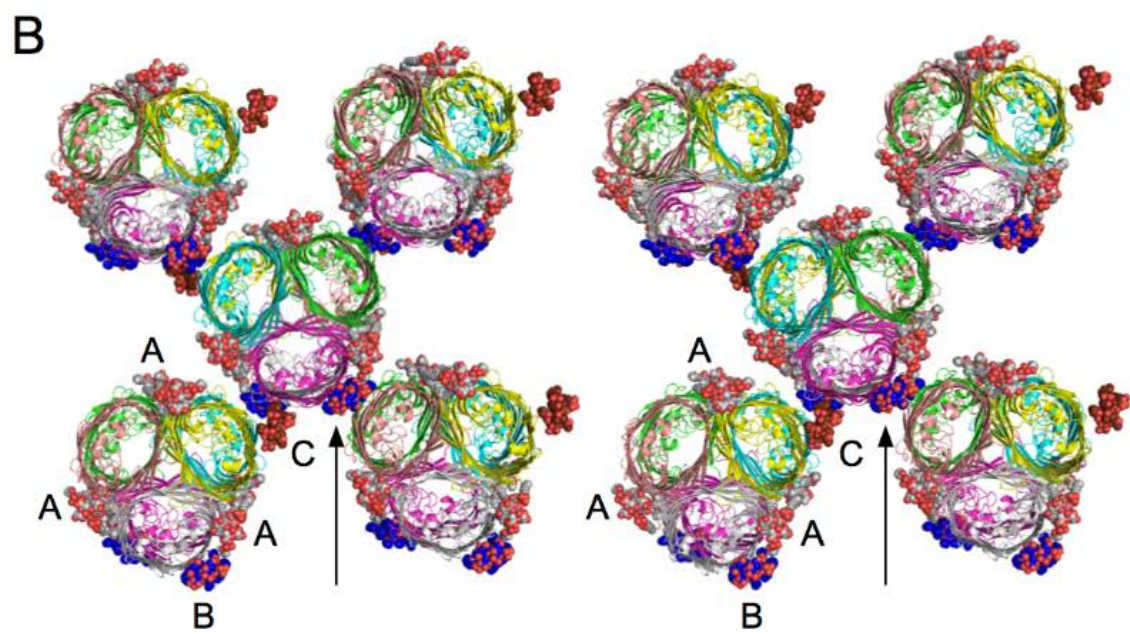
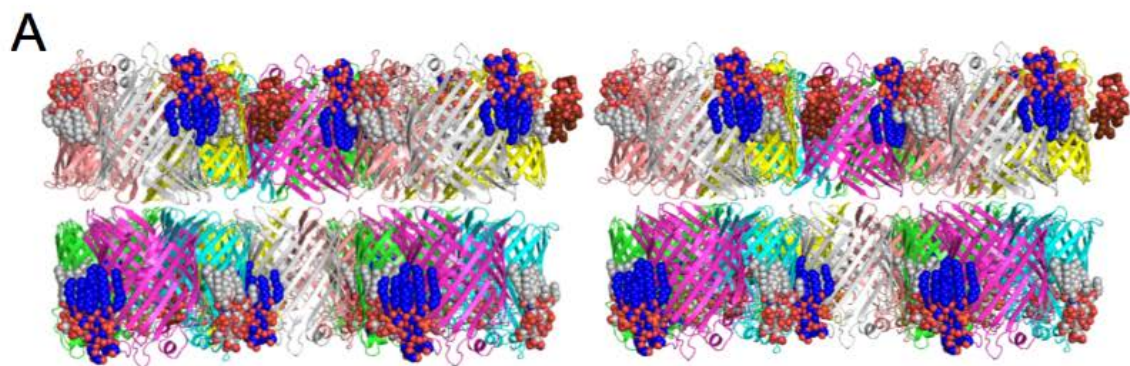


## (D) 8LPS compact model

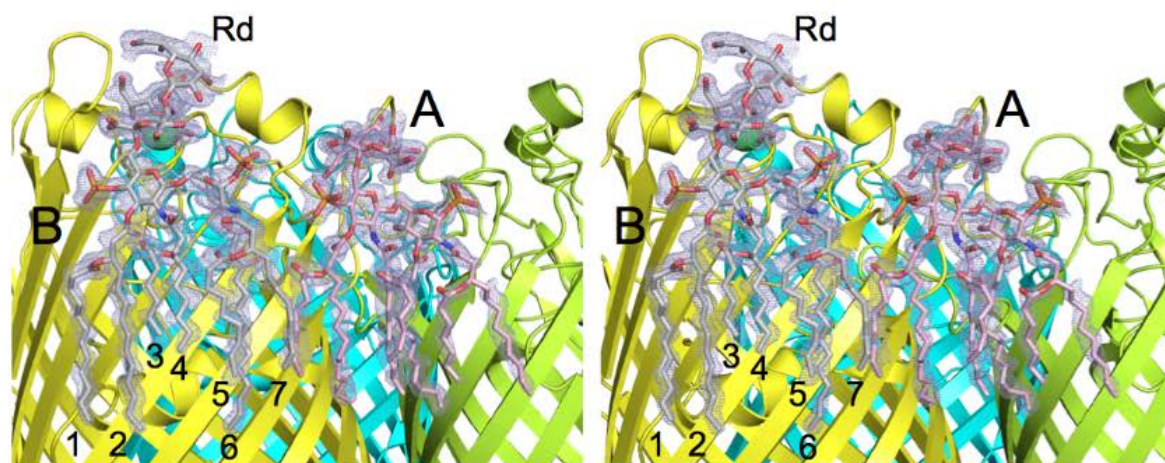




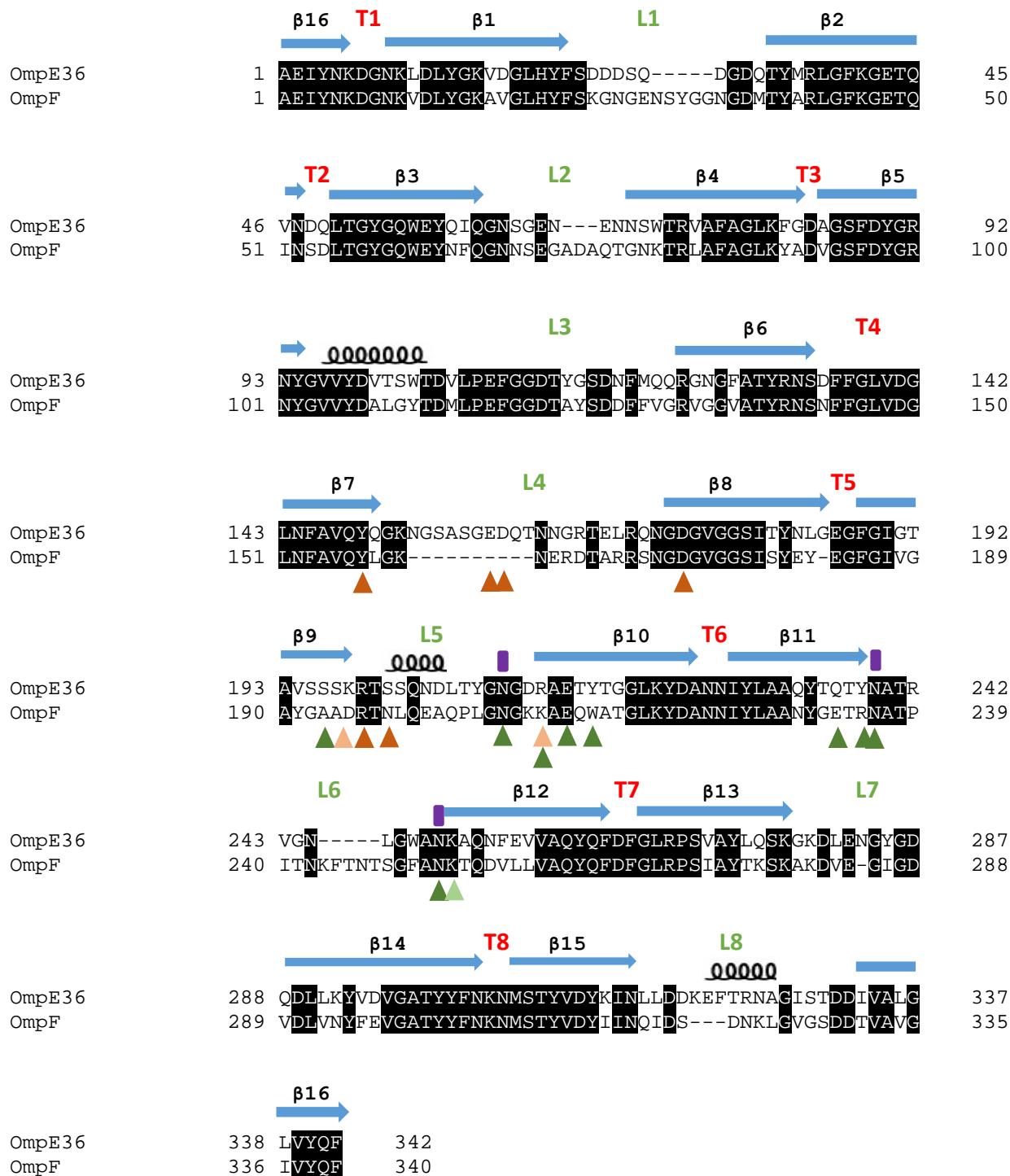
**Figure S3.** SANS Data. (A) Plots of  $\sqrt{I_0}$ /concentration versus % D<sub>2</sub>O to obtain point of zero intensity = match point for the samples shown. (B) Theoretical calculations for the match point of complexes with different levels of LPS made using the SASSIE contrast calculator. (<https://sassie-web.chem.utk.edu/sassie2/>) (7). (C) Quality of the CRYSON fit ( $\chi^2$ ) comparison for the four models. The data fitting by CRYSON suggests an extended than compact model for the complex. The results are not conclusive regarding whether there 2 or 3 molecules per OmpF monomer; fits are very poor for a complete annulus of 8 LPS per OMP monomer (8). (D) Scattering data modelled by CRYSON for each of the models in (C) (solid lines) compared with experimental scattering data for different neutron contrast data.








**Figure S4** Crystal packing of the OmpE36-LPS structure. Stereoviews from the side (A) and from the top (B), showing the arrangement of LPS molecules in the crystal. In B, the three different LPS sites are indicated (A, B, C), and the arrow highlights the important LPS B site which is sandwiched between two symmetry-related OmpE36 trimers.

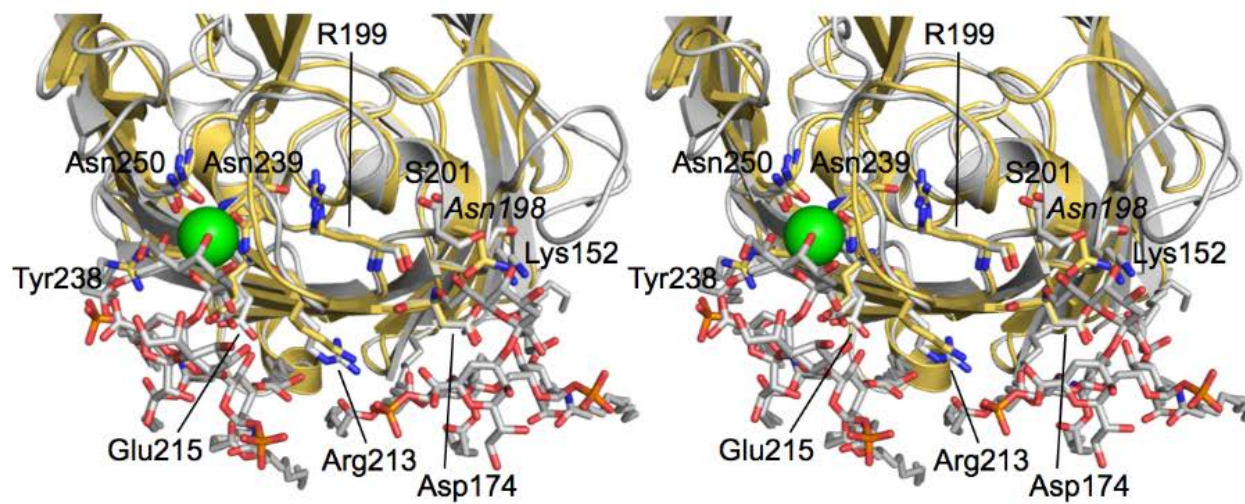


**Figure S5** LPS bound to OmpE36 is hepta-acylated. Stereo view from the side, showing 2Fo-Fc electron density (contoured at 1.0  $\sigma$ ) for LPS A and B. The seven lipid A acyl chains for LPS B have been numbered. The heptose subunit of Rd LPS B is labeled as well. The cartoon models for OmpE36 are colored differently from Fig. 5 to improve contrast with the electron density mesh.



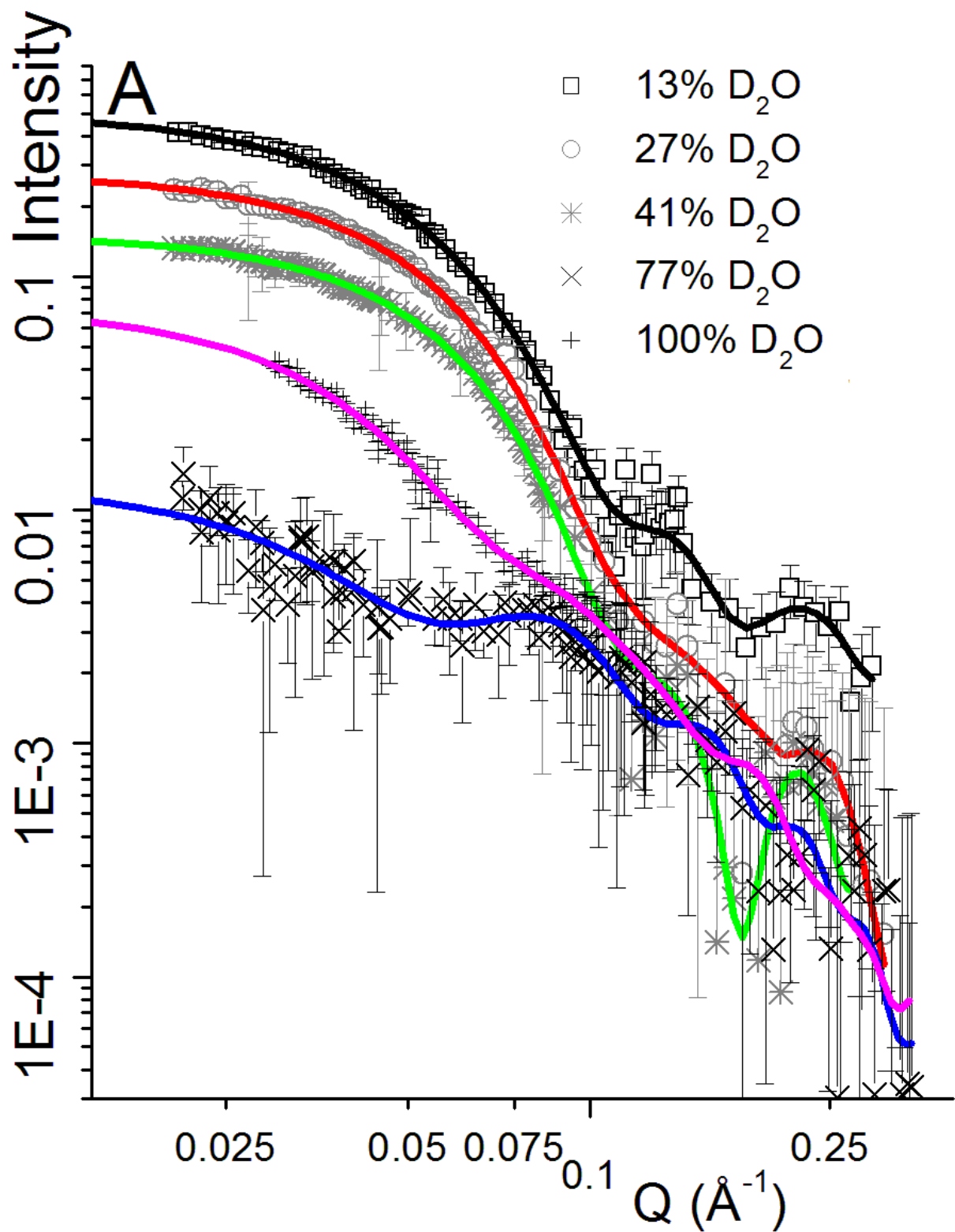
**Figure S6** Comparison of *E. coli* OmpF and OmpE36 from *Enterobacter cloacae*. (A) Sequence alignment of using Clustalw. Conserved residues are highlighted in black. The secondary elements of OmpE36 are indicated as follows: arrows,  $\beta$ -strands; coils,  $\alpha$ -helices; L1-L8, extracellular loops; T1-T8, periplasmic turns. The interactions of LPS moieties (LPS A and LPS B) with one of the monomers of OmpE36 have been shown by labelling the protein residues in following manner:

-  Residue (OmpE36):LPS A interaction; salt bridge (light orange triangle)
-  Residue (OmpE36):LPS A interaction; H-bond (dark orange triangle)
-  Residue (OmpE36):LPS B interaction; salt bridge (light green triangle)
-  Residue (OmpE36):LPS B interaction; H-bond (dark green triangle)
-  Residue (OmpE36):Calcium metal ion ionic interaction (purple bar)





**Figure S7.** The LPS binding sites of OmpE36 and *E. coli* OmpF are similar. Stereo view superposition of OmpE36 (grey) and *E. coli* OmpF (beige), using the program Coot (9), viewed from the extracellular side. Polar residues interacting with LPS A and B are labelled (only for OmpE36 for the sake of clarity). Calcium is indicated by a green sphere. The majority of residues interacting with OmpE36/OmpF are conserved (see also Figure S7).



**Figure S8.** Enlarged version of Figure 4A. In order to show data points more clearly, the same figure presented in the text is reproduced here. The figure legend in the main

text reads “Figure 4. SANS data indicates that LPS binds at the periphery of OmpF in SDS solution. (A) Scattering data for deuterated (d-)OmpF in complex with hydrogenous Ra-LPS after size exclusion chromatography. Fitted lines were generated by the program BayesApp to calculate the  $P(r)$  plots (see Table 1 for fitting parameters and supplementary data for an enlarged Fig. 4A).”

## Supplementary References

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Table 1

**Table 1. Scattering parameters for the SANS data calculated using the program BayesApp (58,59) §**

% D <sub>2</sub> O	$I_0 \times 10^3$	$R_g$ , Å	$D_{max}$ , Å
Deuterated samples (dOmpF-hLPS)			
13	501±0.866	35.48±0.11	116.2±1.71
27	276±0.434	32.6±0.08	91.99±2.05
41	153±0.234	30.68±0.07	76.05±1.68
77	12.7±0.162	42.06±0.46	101.01±1.6
100	73.3±0.94	43.78±0.33	119.56±1.65
Hydrogenated samples (hOmpF-hLPS)			
100	376±1.12	36.84±0.24	121.7±7.99

§  $I_0$  = forward (maximum) scattering intensity at zero angle in arbitrary units;  $R_g$  = radius of gyration and  $D_{max}$  = maximum linear distance across the particle.